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Pathways for flip-flop of mono- and di-anionic phospholipids in the erythrocyte membrane

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Abstract

The inward translocations (flip), from the outer to the inner membrane leaflet of human erythrocytes, of di-anionic NBD-labeled phospholipids containing as a head group phosphate esters of glycolate, butyrate and hydroxyethanesulfonate are slow processes ($k = 0.005–0.008$ h^{-1}, 37°C) at pH 7.4. A decrease of pH highly stimulates the flip. A major role of the anion exchanger (AE1), band 3, in this flip is indicated by (a) the strong inhibition of the flip (55–85%) by stilbene disulfonates and other inhibitors of anion transport, (b) the stimulation and loss of pH dependence of the flip after modification of band 3 by Woodward's reagent K and $NaBH_4$, and (c) the stimulation of the flip after proteolytic cleavage of band 3 by papain. The flip of mono-anionic NBD-phospholipids with phosphate esters of glycerol, glycol, methanol, butanol and benzyl alcohol is much faster than that of their dianionic analogs ($k = 0.04$ to > 3.0 h^{-1}, 37°C). It is inhibited by stilbene disulfonates to a decreasing extent (35 to 0%) and is not affected by several reversible inhibitors of anion exchange. This indicates a minor component of band-3-mediated flip and a major component of nonmediated flip. The outward translocations (flop), from the inner to outer membrane leaflet, of both mono- and di-anionic phospholipids are very fast (1.0–5.9 h^{-1}), ATP-dependent and inhibitable by vanadate, fluoride, SH-reagents or Mg^{2+}-depletion of cells and thereby likely to be largely mediated by a 'floppase'. The stationary distributions of the NBD-labeled anionic phospholipids are asymmetric to an extent (outer to inner leaflet ratio 2–9) correlating with the ratio of the rates of the outward and the inward translocation. Thus, asymmetry is largely abolished by blockage of the floppase-mediated translocation.

Keywords: Erythrocyte membrane; Anionic phospholipid; Flip-flop; Anion transport; Band 3; Flippase

1. Introduction

The inward translocation (flip) of phosphatidylcholine (PC) from the outer to inner membrane leaflet of human erythrocytes is a slow nonmediated process, which probably takes place at structural defects in the hydrophobic barrier [1,2]. The flip of phosphatidylserine (PS) and phosphatidylethanolamine (PE) is a fast, active process requiring ATP and is mediated by a flippase [3], which is specific for these aminophospholipids [4]. Protein mediation and ATP dependence have also been reported for the outward translocations (flop) of both PC [5,6] and inner leaflet phospholipid species [6,7]. Recently, we obtained evidence for an involvement of the anion exchanger, band 3 protein, in the flip of two anionic phospholipids, the monoanionic ^{14}C-labeled lysophosphatidylmethanol (LPM) and the largely dianionic (at pH 7.4), fluorescent, NBD-labeled phosphatidic acid (PA) [8]. Such a mediation by band 3 has also been found for the flip of a long-chain amphiphilic anion 5-(*N*-decyl)aminonaphtalene-2-sulfonate (DENSA) [9]. In the case of the flip of anionic lipids, band 3 acts as a flippase [10], meaning that the anionic head group of the lipid approaches the binding site on band 3

Abbreviations: DENSA, 5-(*N*-decyl)aminonaphtalene-2-sulfonate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; LPM, lysophosphatidylmethanol; NBD-PC, 1-oleoyl-2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino hexanoyl]-*sn*-glycero-3-phosphocholine; NPP, 4-nitrophenylphosphate; PA, phosphatidic acid; PB, phosphatidylbutanol; PBA, phosphatidylhydroxybutyric acid; PBz, phosphatidylbenzylalcohol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PESA, phosphatidylhydroxyethanesulfonic acid; PG, phosphatidylglycerol; PGc, phosphatidylglycol; PGcA, phosphatidylglycolic acid; PM, phosphatidylmethanol; PS, phosphatidylserine; WRK, Woodward's reagent K.

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from the lipid domain instead of the usual access of physiological anions from the aqueous medium.

In the present model study, we investigated the influence of the structure and valence of the anionic head group of the phospholipid on the contributions of the various translocation pathways to the phospholipid translocation from the outer to the inner membrane leaflet and vice versa. To this aim, we prepared a variety of mono- and divalent anionic fluorescent NBD-labeled phospholipids and analyzed the effects on phospholipid translocation of various inhibitors of anion exchange to estimate the contribution of band-3-mediated translocation as well as of ATPase inhibitors to estimate the contribution of ATP-dependent translocation.

2. Materials and methods

2.1. Materials

Heparinized human blood or erythrocyte concentrates were obtained from the local blood bank and used within 8 days. Erythrocytes were isolated by centrifugation. In the case of blood, the buffy coat was carefully removed and the cells were washed three times with isotonic saline.

Incubation media contained (mmol l^{-1}): KCl (90), NaCl (45), sucrose (44) and either phosphate (12.5; pH 7.4), HEPES (10; pH 7.4), MES (30; pH range 5.3–6.5) or TAPS (30; pH range 8.0–8.9) termed KNPS, KNHS, KNMS and KNTS.

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, USA), 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS) from Pfaltz and Bauer (Waterbury, USA), 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonic acid (H_2DIDS) from HSC (Toronto, Canada), 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) from Calbiochem (Bad Soden), eosin-5-maleimide from Molecular Probes (Eugene, USA) and 4-hydroxybutyric acid, phenylarsine oxide as well as 2-hydroxyethanesulfonic acid from Aldrich (Steinheim). Phospholipase D from Streptomyces species, papain, pyridoxal-5-phosphate, Woodward's reagent K (WRK), phloretin, serine, ethanolamine and dipyridamole were purchased from Sigma (Deisenhofen). Alkaline phosphatase, *N*-ethylmaleimide and 4-nitrophenylphosphate (NPP) were obtained from Fluka (Neu-Ulm). Benzyl alcohol, glycolic acid, methanol, n-butanol and ethylacetate were purchased from Merck (Darmstadt).

2.2. Synthesis of anionic NBD-phospholipids

In the presence of a single defined alcohol, phospholipase D has been demonstrated [4,11–13] to catalyze the exchange of the choline residue of PC for the alcohol and to produce a single transphosphatidylation product with variable amounts of the hydrolysis product phosphatidic acid. We now synthetized NBD-phospholipids with various polar head groups essentially according to Juneja et al. [13]. Briefly, 0.5 mg of NBD-labeled PC was dissolved in 40 μl of chloroform and mixed with 600 μl of ethylacetate. To prepare NBD-PA, 300 μl of a solution containing MES (0.1 mmol l^{-1}, pH = 5.6) and $CaCl_2$ (0.1 mmol l^{-1}) was added. To prepare NBD-labeled phosphatidylmethanol (PM), phosphatidylbutanol (PB), phosphatidylglycol (PGc), phosphatidylglycerol (PG), phosphatidylglycolic acid (PGcA), phosphatidylbutyric acid (PBA), phosphatidylhydroxyethanesulfonic acid (PESA), phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylbenzylalcohol (PBz), 300 μl of a solution containing MES, $CaCl_2$ and resp. 3 mmol l^{-1} methanol, butanol, glycol, glycerol, glycolic acid, 4-hydroxybutyric acid, 2-hydroxyethanesulfonic acid, serine, ethanolamine or 0.5 mmol l^{-1} benzyl alcohol was added. After addition of 2.5 units of phospholipase D, the mixtures were shaken for 30 min at 30°C, the enzymatic activity was blocked by addition of 360 μl of 250 mmol l^{-1} EDTA and the organic solvent layer evaporated under a stream of N_2. The aqueous layer was then mixed with 180 μl of 0.02 N HCl (final pH 3–4) and extracted with 1 ml of dichloromethane. The dichloromethane layer was removed and the extraction repeated. The combined extracts were washed with 400 μl of 0.02 N HCl and evaporated. The desired product was then purified by thin-layer chromatography on silica gel 60 plates (Merck, Darmstadt), which had been impregnated with 2.3% H_3BO_3 in ethanol and dried at 110°C. In the case of NBD-labeled PA, PM, PE, PS, PGc and PG, the plates were developed with a solvent mixture containing chloroform/ethanol/H_2O/triethylamine (25:35:6:35), in the case of NBD-labeled PGcA and PESA with chloroform/methanol/H_2O/96% acetic acid (30:15:1:6) and in the case of NBD-PB, NBD-PBz and NBD-PBA with chloroform/methanol/H_2O/NH_3 (45:22.5:2.6:2.8). The solvent systems used gave complete separations between traces of the starting material NBD-PC, the single transphosphatidylation product and the hydrolysis product NBD-PA, which was confirmed by rechromatography of the product using various solvent systems and running reference phospholipids in parallel lanes. The fluorescent spot containing the desired phospholipid was scraped from the plate and the phospholipid extracted from the silica by three 2-ml portions of dichloromethane/methanol (2:1) followed by two 2-ml portions of dichloromethane/methanol/H_2O (2:5:2). The combined extracts were evaporated to dryness, the NBD-phospholipid was dissolved in ethanol (final concentration 0.2 mmol l^{-1}) and residual silica removed by centrifugation.

2.3. Modification of erythrocytes

Erythrocytes were pretreated with DIDS, H_2DIDS, eosin-5-maleimide (30 min, 37°C, pH 7.4) or with 2 mmol

l^{-1} Woodward's reagent K (WRK) followed by 4 mmol l^{-1} $NaBH_4$ (both 10 min, 0°C, pH 7.0) in KNHS and washed 3 times with medium or in the case of eosin-5-maleimide two times with KNPS containing 0.2 g.dl^{-1} albumin followed by two washes with KNPS. Exofacial proteolytic cleavage of band 3 was carried out by treatment of erythrocytes in KNMS (50% Hct) with cysteine-activated papain (30 U/ml cells, 60 min, 37°C, pH 6.0), followed by two washes with 0.2% albumin in KNPS and two washes with KNPS.

2.4. ATP depletion of cells

In order to deplete cells of ATP to about 5% of original level (data not shown, Ref. [14]), cells were treated with 5 mmol l^{-1} or in some cases 0.2 mmol l^{-1} iodoacetate in KNPS (15 min, 37°C, pH 7.4), washed with KNPS, incubated with 5 mmol l^{-1} inosine (45 min, 37°C, pH 7.4) and washed with KNPS.

2.5. Measurement of inward translocation (flip) of NBD-phospholipids

Trace amounts of fluorescent NBD-phospholipid added as an ethanolic solution were inserted into the outer membrane leaflet of erythrocytes (7 min, 0°C) and cells washed once. The ratio NBD-phospholipid/membrane phospholipid was about 1:500. Subsequently, cells were resuspended in medium (Hct 10%) and incubated at 37°C unless otherwise indicated. To measure the time-dependent flip by the increase of fluorescence in the inner membrane leaflet, 100 μl samples of the suspension were taken at various time periods, mixed with 550 μl medium containing 1.5 g dl^{-1} albumin, incubated (2 min) and centrifuged. The albumin treatment was repeated once and cells were washed with medium to remove residual albumin. Cells were then hemolyzed with 100 μl of water, followed by lipid extraction with 800 μl of isopropanol. After centrifugation of the extracts, fluorescence was measured at 522 nm (excitation at 466 nm) using a Shimadzu spectrofluorophotometer (RF5001 PC). The fluorescence remaining after albumin extraction (probe in inner membrane leaflet, P_i) was related to the total isopropanol-extractable fluorescence, P_{tot}, in the same amount of hemolyzed cells. An exponentional function, $q \cdot [1 - \exp(-k_1 \cdot t/q)]$, was fitted to the kinetic data of P_i/P_{tot}, where q represents the fraction of probe in the inner leaflet under stationary conditions and k_1 is the rate constant for the unidirectional flip.

In some very fast translocations, the fraction of the probe in the inner leaflet was derived from its non-reducibility by dithionite according to the method of McIntyre and Sleight [15]. To this extent, 100 μl of the erythrocyte suspension were incubated (2 min, 0°C) with 700 μl of a solution containing 80 mmol l^{-1} dithionite and 80 mmol l^{-1} Tris (pH 10). Following centrifugation, cells were washed with medium (0°C) and NBD-phospholipids extracted with isopropanol as described above.

2.6. Measurement of outward translocation (flop) of NBD-phospholipids

Following flip (60–90 min) of the NBD-phospholipid, the probe was removed from the outer leaflet by two repetitive albumin (1.5 g dl^{-1}) extractions. After washing of the cells with KNPS to remove albumin, the flop of the NBD-phospholipid was followed by measuring the time-dependent decrease of albumin-inextractable fluorescence. To fasten the slow accumulation of NBD-PC in the inner leaflet, its flip was accelerated reversibly by addition of 1 mol l^{-1} ethanol [16]. In the case of NBD-PGcA, the flip was carried out at pH 6.0. An exponentional curve, $(1 - q) \cdot \exp[-k_{-1} \cdot t/(1 - q)] + q$, was fitted to the kinetic data of the non-extractable fractions (P_i/P_{tot}), where k_{-1} is the rate constant for the unidirectional flop.

2.7. Measurement of transport of 4-nitrophenylphosphate (NPP)

To measure the uptake of NPP by erythrocytes, cells were pretreated (20 min, 37°C) with 5 mmol l^{-1} *N*-ethylmaleimide to block endogenous phosphatase activity. After washing of the cells with KNPS (pH 7.4) or KNMS (pH 6.0) and centrifugation, 1 ml of packed cells were mixed with 1.5 ml of KNPS or KNMS containing 0.75 mmol l^{-1} NPP and the time-dependent disappearance of NPP from the medium was followed. To this end, 250 μl of the suspension were centrifuged and 130 μl of the supernatant incubated (15 min) with 3.25 μl of $HClO_4$ (60%). After centrifugation, 100 μl of the supernatant were mixed with 900 μl of diethanolamine buffer (1 mol l^{-1}; pH 8.9) containing $MgCl_2$ (0.25 mmol l^{-1}) and alkaline phosphatase (80 U). The mixture was then incubated for 10 min and the absorbance read at 410 nm.

3. Results and interpretation

3.1. Kinetics of translocation

In the present study, a number of monovalent and divalent anionic NBD-labeled phospholipids containing as a head group phosphate esters of various neutral and anionic molecules (Fig. 1) was prepared from NBD-labeled PC by well established [4,11–13] enzymatic transphosphatidylation using phospholipase D. Following insertion of the NBD-labeled phospholipid into the outer membrane leaflet, its time-dependent translocation to the inner leaflet was measured and a single exponentional fitted to the data, from which the rate constant k_1 for the unidirectional translocation and the fraction of probe in the inner leaflet under stationary conditions, q (see Section 2), were derived.

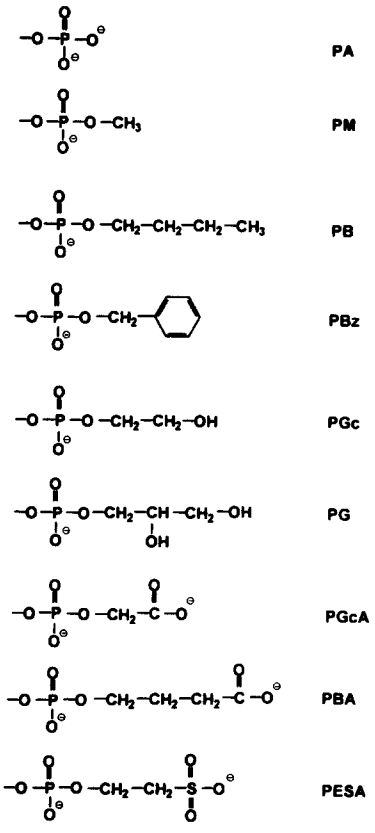

Fig. 1. Structures of the polar head group of the anionic NBD-phospholipids used.

As shown in Fig. 2, the rate of the inward translocation (flip) of the NBD-phospholipids, at pH 7.4, highly depends on the structure and valence of the polar moiety of the phospholipid (pK_a of the phosphate group about 2; Ref. [17]). The monoanionic phosphatidylmethanol (PM) with its small head group containing the non-polar methyl residue moves inward very rapidly with a half-time of less than 5 min corresponding with a rate constant (k_1) of about 2.6 h^{-1} (37°C, pH 7.4; Table 1). The flip of the lyso-analogue of NBD-PM, ^{14}C-labeled lysophosphatidyl-

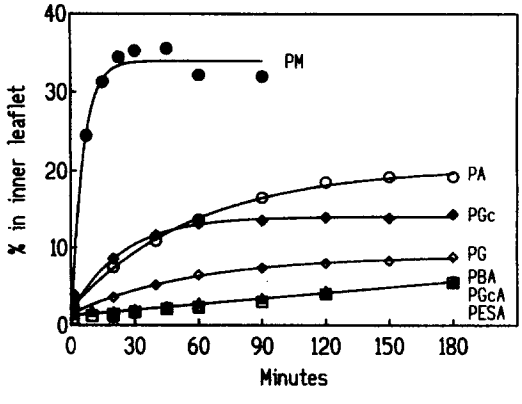

Fig. 2. Translocation of NBD-labeled PM, PA, PGc, PG, PGcA, PBA and PESA to the inner membrane leaflet at 37°C following their insertion into the outer leaflet.

Table 1
Rate constants (k_1) for the inward translocation (flip) of various anionic NBD-labeled phospholipids in erythrocytes with different metabolic status

Phospholipid	Control	k_1 (h^{-1}) [a]	
		supplemented [b]	ATP-depleted
Monoanionic			
PM	2.63	3.01 ± 0.71 (5)	3.25
PGc	0.15 ± 0.04 (14)	0.27	0.26
PG	0.038 ± 0.005 (11)	0.060	0.040
Dianionic			
PA	0.17 ± 0.02 (18)	0.19	0.19 ± 0.02 (9)
PGcA	0.008		
PBA	0.006		
PESA	0.005		
PS	1.72 ± 0.36 (14)		
PC	0.02		

[a] Mean values for 2–3 experiments or ± standard deviation for the number of experiments (37°C, pH 7.4) given in parentheses
[b] Flip was measured in the presence of 5 mmol l^{-1} inosine or glucose.

methanol (LPM), is about 3-fold slower [8]. Elongation of the alkyl chain of the head group alcohol to 4 carbon atoms (NBD-PB) enhances the flip. Kinetics of NBD-PB flip are too fast to determine the rate constant at 37°C. At 15°C, it has a rate constant of about 1 h^{-1} (data not shown), which is about 5-fold higher than that of NBD-PM (0.2 h^{-1}, 15°C). Another NBD-phospholipid with the head group containing the apolar benzyl residue also has a high flip rate (0.8 h^{-1}, 15°C). The translocations (37°C) of the monoanionic probe, NBD-phosphatidylglycol (PGc), with one hydroxyethyl group on its polar head, and of NBD-phosphatidic acid (PA), which is largely dianionic (pK_{a2} in the membrane about 6.3, Ref. [17]), are about 10-fold slower (37°C) under the conditions of our experiments than the translocation of NBD-PM. The monoanionic NBD-phosphatidylglycerol (PG) with a dihydroxypropyl group on its polar head moves about 50-fold slower and the dianionic phospholipids, NBD-phosphatidylglycolic acid (PGcA), NBD-phosphatidylbutyric acid (PBA) (pK_a of the carboxyl group about 3; Ref. [17]) and phosphatidylhydroxyethanesulfonic acid (NBD-PESA) (pK_a of the sulfonic acid below 2), even about 300-fold slower than NBD-PM (Fig. 2 and Table 1). Thus, an increase of polarity and charge number of the phospholipid head group decreases its flip rate. ATP depletion (see Section 2) has no inhibitory effect on the flip rates of the probes (Table 1). This excludes a contribution of the ATP-dependent aminophospholipid flippase [3] to their flip.

The kinetics shown in Fig. 2 indicate a preference of NBD-labeled PM, PGc, PG and PA for the outer membrane leaflet of the cells after attainment of stationary conditions. This preference also becomes evident from the kinetics of the corresponding outward translocation (flop) obtained as described in Section 2 (Fig. 3). Interestingly, the flop rate constants calculated by non-linear regression

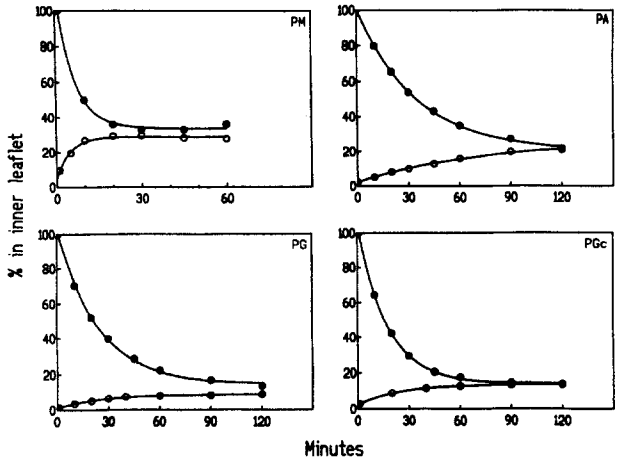

Fig. 3. Translocation of NBD-labeled PM, PGc, PG and PA to the outer membrane leaflet at 37°C (closed circles) following their translocation to the inner leaflet (open circles).

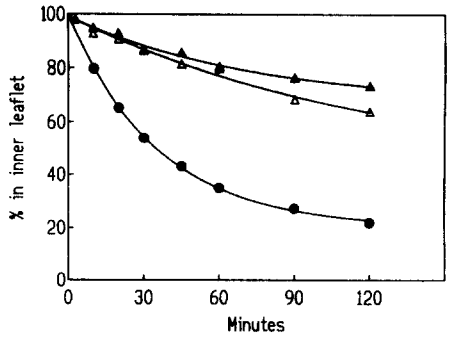

Fig. 4. ATP-dependence of the outward translocation of NBD-labeled PA. Translocation kinetics for ATP-containing cells (filled circles), cells in presence of 6 mmol l^{-1} KF (filled triangles) and ATP-depleted cells (empty triangles, see Section 2).

are considerably higher (Table 2) than those for the corresponding flip (Table 1). This property facilitated the estimation of the stationary distribution of the NBD-phospholipids between the inner and outer leaflet. From both inward and outward kinetics, fractions of NBD-labeled PM, PG, PGc and PA in the inner leaflet, under stationary conditions, of 0.36, 0.10, 0.14 and 0.23 were derived (Table 2).

A sequential incubation of cells with iodoacetate and inosine (see Section 2) resulting in ATP-depletion decreased the rates of flop of the anionic phospholipids by 70–90% (Fig. 4 and Table 2), while the flip was not significantly affected (Table 1). Inhibition of flop by the inosine incubation after the treatment with iodoacetate and its removal indicates a causal role of ATP-depletion (Fig. 4). Moreover, the flop was suppressed by addition of 0.5 mmol l^{-1} orthovanadate (data not shown) or 6 mmol l^{-1} fluoride (Fig. 4), by Mg^{2+}-depletion of cells and by a pretreatment of cells with the SH-reagents, *N*-ethylmaleimide or phenylarsine oxide (resp. 0.8 and 0.1 mmol l^{-1}, 15 min, 37°C; data not shown). These findings clearly suggest the major involvement of an ATPase in the flop process. An ATP dependence of the flop of phospholipids has previously been reported for NBD-labeled PC, PE and PS [7]. The outward translocations remaining after ATP-depletion have rate constants comparable to those of the corresponding inward translocations (Tables 1 and 2).

ATP-depletion not only lowers the rates of flop, but also shifts the asymmetric distribution of the anionic phospholipid probes, observed in cells with normal ATP levels, to a more or less symmetric distribution (Table 2). The changes of the stationary distributions of the phospholipid probes in ATP-depleted and ATP-containing cells thus correlate qualitatively with the changes of the ratio of the rate constants for the inward and outward phospholipid translocations.

Table 2
Rate constants (k_{-1}) [a] for the outward translocation (flop) of various NBD-labeled phospholipids and the fractions of the probes in the outer membrane leaflet under stationary conditions (q) [b]

Phospholipid	Control cells		ATP-depleted cells	
	k_{-1} (h^{-1})	q	k_{-1} (h^{-1})	q
PM	5.92	0.36	1.90	0.65
PGc	2.83	0.14	0.23	0.56
PG	1.77	0.10		
PA	1.44	0.23	0.24±0.06	0.45±0.05
PGcA	1.05			
PC	0.76±0.19	0.25	0.05	
PS	0.47			
PE	0.44			

[a] Rate constants for the flop (37°C, pH 7.4) in control and ATP-depleted cells represent mean values for 2–3 experiments or ± standard deviation for 11 and 6 experiments.

[b] Mean values of the fractions of phospholipid in the inner membrane leaflet under stationary conditions following flop and/or flip of the phospholipid represent mean values for 2–3 experiments or ± standard deviation for 9 experiments.

3.2. *Inhibition of flip by stilbene disulfonates*

The flip of the various anionic NBD-phospholipids can be inhibited by stilbene disulfonates, which are potent inhibitors of anion exchange via band 3 [18]. A pretreatment (30 min, 37°C, pH 7.4) of cells with H_2DIDS or DIDS (10 μmol l^{-1}), known to specifically block anion exchange at these low concentrations [18], as well as by the presence of DIDS or DNDS during flip, suppress the translocations of the various NBD-phospholipids, albeit to a variable extent (Table 3). The extent of inhibition by the stilbene disulfonates is higher (50–85%) in the case of the dianionic NBD-phospholipids (PA, PBA, PGcA and PESA) than in that of the monoanionic NBD-phospholipids (from 35% for NBD-PG to 0% for NBD-PB; Table 3). Higher concentrations of the inhibitors do not increase the inhibition. These results provide evidence for an involvement of the anion exchanger band 3 (AE1) in the flip of the dianionic NBD-phospholipids and, to a lesser extent of the

monoanionic NBD-phospholipids, PG, PGc and PM. In ATP-depleted cells, the inhibition by DIDS of the flip of NBD-PA is somewhat higher (76%, Table 3), which is probably related to the suppression of its 7-fold faster flop.

The incomplete inhibition of the flip of anionic phospholipids by DIDS may be explained in two ways [8]. Firstly, flip of the anionic phospholipids could involve two parallel pathways of which one is via band 3, the other not. Secondly, contrary to the exchange of hydrophilic anions, the inhibition by DIDS of the band-3-mediated flip of anionic phospholipids might be incomplete [8]. To distinguish between these two possibilities, further characteristics of the translocation of the NBD-phospholipids were studied.

3.3. pH dependence of flip

Band-3-mediated transport of small monovalent inorganic anions and of divalent inorganic or organic anions exhibit strong pH dependencies with maxima respectively at or above pH 7 [19–21] and at or below pH 6.5 [20–25] (also, Deuticke, personal communication). The flip of the monoanionic NBD-phospholipids, PG, PGc and PM as well as that of NBD-PA exhibit a small pH dependence (Fig. 5). A decrease of pH from 8.5 to 6.5 results in a 2- to 3.5-fold increase of the flip rate. Although this small pH dependence differs from that of band-3-mediated transport of chloride, it corresponds to the small pH dependence of the band-3-mediated flip of the monoanionic lysophospolipid, ^{14}C-LPM [8] and the monoanionic amphiphile 5-(*N*-decyl)aminonaphtalene-2-sulfonate (DENSA; Ref. [9]).

Table 3
Effects of various stilbene disulfonates on the inward translocation of anionic NBD-labeled phospholipids

Phospholipid	% Inhibition [a]			
	H_2DIDS [b]	DIDS	DNDS	DIDS(-ATP) [c]
Monoanionic				
PB			0	
PM	20	21	15	20
PGc	33	34	16	27
PG	49	36	37	35
Dianionic				
PA	66±7 [d]	65	63	76.3±2.7 [b]
PGcA	81 [f]	76 [f]	74 [e]	
PBA	57 [g]		49 [e]	
PESA			87 [e]	

[a] Mean values of 2–3 experiments or mean values ± standard deviation for 8 experiments.
[b] Pretreatment with H_2DIDS or DIDS (30 min, 37°C), otherwise 50 μmol l^{-1} DIDS or 500 μmol l^{-1} DNDS during translocation measurement.
[c] ATP-depleted cells.
[d] Ref. [8].
[e,f,g] Due to the slow flip rates of dianionic phospholipids at pH 7.4, the pH during flip was respectively 5.5, 6.0 and 6.5.

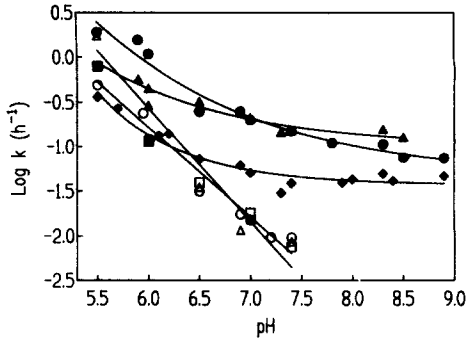

Fig. 5. pH dependence of the inward translocation of NBD-labeled PA (filled circles), PGc (filled triangles), PG (filled diamonds), PBA (empty squares), PGcA (empty circles), and PESA (empty triangles). Erythrocytes were titrated to the extracellular pH given and the translocations measured at the corresponding pH in buffered media (see Section 2).

On the other hand, the flip of the dianionic phospholipids is highly pH-dependent (Fig. 5). Flip increases about 10-fold upon a decrease of pH by one unit. This suggests the titration of a weakly dissociated moiety either on the phospholipid probe or the flip site. The pH dependence of flip of dianionic NBD-phospholipids is in line with that of transport of divalent hydrophilic anions, which was attributed to the protonation of an amino acid residue with a pK_a value of about 5.5 [20,24]. In the following, evidence for an involvement of the protonation of a glutamate residue of band 3 in the pH dependence of the translocation of dianionic phospholipids will be presented.

3.4. Effect of Woodward's reagent K (WRK) on flip

A mild treatment of erythrocytes with WRK/BH_4^- (see Section 2), which converts the glutamate residue at position 681 of the human band 3 protein to the corresponding alcohol [26], has been shown to accelerate the self-exchange of divalent anion sulfate while inhibiting the self-exchange of the monovalent anion chloride [25,26]. As expected for the transport of a divalent anion by band 3, the flip at pH 7.4 of the dianionic NBD-phospholipids, PGcA and PESA, with two spatially separated, but rather closely adjacent, negative charges at the polar head group is highly accelerated. In contrast, the flip of PBA with a larger separation between the two negative charges is accelerated only moderately (Table 4). These results are in line with our previous findings [8], that the flip of NBD-PA, largely dianionic at physiological pH [17], is strongly accelerated by WRK/BH_4^-.

A more detailed study demonstrated that WRK/BH_4^- treatment completely or partly abolishes the pH dependence of the flip of NBD-labeled PA, PESA and PBA (Fig. 6). A similar phenomenon has been reported for the transport of sulfate [25]. Since the pK_a values of the carboxylate and sulfonate residues of our NBD-phospholipids are well below the range of pH values studied here and the translocations are strongly suppressed by DIDS up

Table 4
Stimulation of the inward translocation (k/k_0)[a] of various anionic NBD-labeled phospholipids by papain and WRK/BH_4^-

Phospholipid	k/k_0		
	Papain	WRK/BH_4^-	WRK/BH_4^- then papain
Monoanionic			
PM	1.0	1.0	
PG	1.8	1.0	2
PGc	1.5	1.6	2
Dianionic			
PA	5.5	6.0	25
PGcA	6.8	17.0	
PBA	1.7 [b]	2.6 [c]	
PESA	6.9 [b]	6.6 [c]	

[a] k and k_0 are the rate constants for the translocation in treated and untreated cells and k/k_0 values are mean values for 2–4 experiments.
[b,c] The translocation was measured at respectively pH 6.35 and 7.0, instead of 7.4.

to the lower end of the pH range (pH 6.0, Table 3), the observed pH dependencies of the dianionic phospholipids in unmodified cells must reflect a property of the translocation protein, band 3, and not of the transported probe.

While the flip of the dianionic NBD-phospholipids is markedly enhanced, the flip of the monoanionic NBD-phospholipids is slightly accelerated or not affected by WRK/BH_4^- (Table 4), which contrasts to the inhibition of the band-3-mediated flip of two other monoanionic amphiphiles, ^{14}C-LPM and DENSA, by WRK/BH_4^- [8,9]. A major contribution of band 3 to the translocation of monoanionic NBD-phospholipids is therefore unlikely.

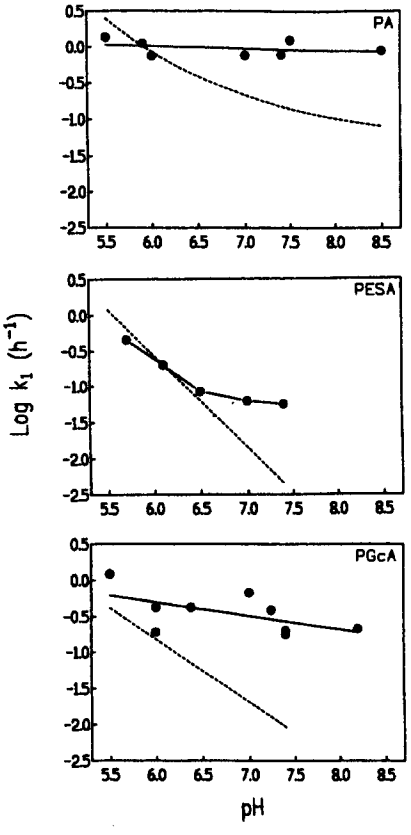

Fig. 6. The effect of WRK/BH_4^- on the pH dependences of the inward translocations of NBD-labeled PA, PGcA and PESA. Erythrocytes pretreated with WRK/BH_4^- (filled circles) and untreated cells (dotted line) were titrated to the extracellular pH given and the translocations measured at the corresponding pH in buffered media (see Section 2).

3.5. Effect of papain on flip

A papain treatment of erythrocytes, which cleaves the band 3 protein at three positions and produces two large and two small fragments [27], has been shown to inhibit the self-exchange of chloride, sulfate and phosphate [28,29] and to accelerate the inward movement of anions under conditions where the ratio of the rates of the outward and the inward movements of the anion exchanger are high [30]. The papain treatment accelerates the flip of dianionic phospholipids (Table 4), which is evidence for an involvement of band 3. On the other hand, papain has no or small stimulating effects on the flip of monoanionic NBD-phospholipids. This contrasts to the strong acceleration of the band-3-mediated flip of monoanionic ^{14}C-LPM and DENSA [8,9].

3.6. Effects of noncovalent inhibitors on flip

The involvement of band 3 in the flip of the dianionic NBD-PGcA is also evident from the inhibitory effects (Table 5) of a number of well established inhibitors of anion exchange via band 3, e.g., salicylate, dipyridamole and tetrathionate [22,31,32]. Inhibitors were used at concentrations that have been reported to highly suppress the exchange of hydrophilic anions [22,31,32]. Flip had to be measured at pH 6.0, instead of 7.4, to increase the flip rate of NBD-PGcA and enable demonstration of inhibition. Flip of NBD-PA at pH 7.4 has previously been found to be inhibited by these inhibitors too [8].

Table 5
Effect of inhibitors of anion exchange on the inward translocation of NBD-labeled PGcA and PA at pH 6.0 as well as on transport of NPP at pH 6.0 and 7.4

Inhibitor (mmol l^{-1})	% Inhibition [a]			
	PGcA	PA [b]	NPP	
	pH = 6.0	6.0	6.0	7.4
DIDS [c] (0.01)	81		99	98
DIDS (0.05)	76	79		100
DNDS (0.5)	74	61	95	
Dipyridamole (0.02)	42	49	63	84
Tetrathionate (25)	69	53		92
Salicylate (40)	39	0 [d]	69	95
Niflumate (0.055)	0	0	51	70
Phloretin (0.5)	0	0 [d]	32	45

[a] Mean values of 2–3 experiments.
[b] ATP-depleted cells.
[c] Pretreatment (30 min, 37°C).
[d] Stimulation, see Fig. 7.

Interestingly, two other inhibitors of anion exchange, niflumate [33] and phloretin [22,34,35], do not affect the flip of NBD-PGcA at pH 6.0. At this pH, the flip of NBD-PA is also not inhibitable by niflumate, while it is accelerated by phloretin and salicylate. From Fig. 7, it becomes evident that the inhibitory effect of these inhibitors is highly pH-dependent. At pH 8.3 the extent of inhibition by niflumate, salicylate and phloretin is resp. 70, 74 and 32%, while at pH 6.0 they produce no inhibition or even acceleration. Furthermore, the inhibitor dipyridamole [36] is less effective at pH 6.0 (49%) than at 8.3 (81%). A small pH-dependent effect of these inhibitors is also observed (Table 5) for the transport of the small amphiphile 4-nitrophenyl phosphate (NPP, see Section 2), which is transported via band 3 as can be derived from its blockage by DIDS (Table 5).

In contrast to the decreased inhibition, by niflumate, salicylate and phloretin, of the flip of dianionic phospholipids when the pH is decreased from 7.4 to 6.0, the extent of inhibition of NBD-PA flip by DIDS is rather pH-independent (Fig. 7). The lack of effect of phloretin on NBD-PA flip at pH 7.4 (Table 6) is therefore postulated to result from the pH-dependent inhibition of band-3-mediated flip (major component) and the pH-independent enhancement of the nonmediated flip (minor component). In line with this idea, a 2- to 3-fold acceleration of flip by 0.5 mmol l^{-1} phloretin is observed for the DIDS-insensitive flip of NBD-PA at pH 7.4 (data not shown). Accelerating effects of phloretin could therefore be due to an increase of flip via a nonmediated pathway analogous to the stimulation by alcohols of phospholipid flip [16] and diffusion of nonelectrolytes via the membrane lipid domain [37]. A band-3-independent mechanism of the DIDS-insensitive flip of NBD-PA is also supported by the lack of effect of changes of the anion milieu on this flip, whereas the relative rate of DIDS-sensitive flip is altered by an exchange of intra- and extracellular Cl^- for NO_3^-, Br^- and SO_4^{2-} from 1.0 to respectively 0.72, 1.2 and 2.3. Similar anion effects were reported for the exchange of inorganic phosphate [22].

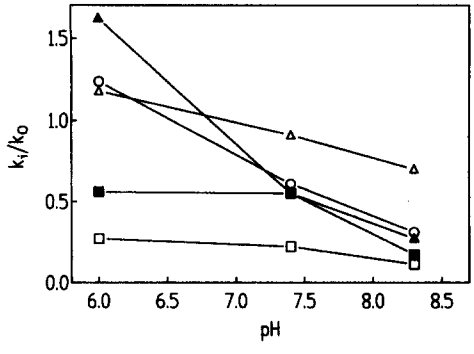

Fig. 7. Effect of pH on the suppression of the inward translocation of NBD-PA by inhibitors of anion exchange: DIDS (empty squares), dipyridamole (filled square), salicylate (filled triangle), niflumate (empty circles) and phloretin (empty triangle). k_i and k_0 are the flip rate constants in the presence and absence of inhibitor.

Table 6
Alteration of the inhibitor effectivity following stimulation of inward translocation of NBD-PA by papain (5.5-fold), WRK/BH_4^- (6-fold) or WRK/BH_4^- then papain (25-fold)

Inhibitor (mmol l^{-1})	Inhibition [a]			
	control	papain [b]	WRK [c]	WRK/papain
DIDS [e] (0.010)	65	85	95	
DIDS (0.050)	65	89	95	84
DNDS (0.500)	63 [d]	73	90	82
Dipyridamole (0.020)	47 [d]	49	15	0
Pyridoxal phospate (30)	45 [d]	0 [f]	89	
Tetrathionate (25)	52 [d]	0	94	83
Salicylate (40)	34 [d]	27	93	34
Phloretin (0.500)	0 [d]	0 [f]	51	0 [f]
Niflumate (0.025)	35 [d] (50 [g])	0	89	0
Erythrosin (0.020)	28	0 [f]	86	
Eosinmaleimide [e](0.100)	76		94	

[a] % inhibition are mean values of 2–4 experiments.
[b] Pretreatment with papain (1 mg/ml of cells, pH 6.0, 60 min, 37°C).
[c] Pretreatment with 2 mmol l^{-1} WRK at pH 7.0 (10 min, 0°C) followed by 4 mmol l^{-1} $NaBH_4$ (10 min, 0°C).
[d] Ref. [8].
[e] Cells pretreated (30 min, 37°C, pH 7.4).
[f] Stimulation.
[g] At 0.075 mmol l^{-1}.

In contrast to the flip of the dianionic NBD-phospholipids, the flip of the monoanionic NBD-phospholipids, PM, PG and PGc, is not affected by salicylate, tetrathionate and dipyridamole (data not shown), is stimulated 2- to 3-fold by phloretin irrespective of the pH and is not affected by changes of the anion milieu (data not shown). This supports the idea that band 3 plays only a minor role in the translocation of monoanionic NBD-phospholipids, as was already derived from the small or moderate inhibitory effect of DIDS and the lack of inhibitory effect of WRK/BH_4^- on translocation (Table 3).

3.7. Effect of modification of band 3 by papain and WRK/BH_4 on flip inhibition

The pattern of effectiveness of inhibitors of anion exchange on the flip of NBD-PA is characteristically altered after modification of cells by papain or WRK/BH_4^-, which markedly accelerate flip. The rate constant (k_1) of flip (pH 7.4, 37°C) increases from 0.17 for control cells to resp. 1.11 and 1.09 h^{-1} for papain and WRK/BH_4^- treated cells (see Section 2). The extent of inhibition of flip (Table 6) by all inhibitors, except dipyridamole, is increased after WRK/BH_4^-. The inhibition of flip by stilbene disulfonates and another inhibitor of anion exchange, eosin-5-maleimide [38] are very high, namely 90–95%. The flip remaining after the treatments with DIDS or eosin-5-maleimide have rate constants comparable to the rate constant for untreated cells in the presence of DIDS. This means that the inhibitors completely suppress the flip induced by WRK/BH_4^-.

Following a papain treatment, the inhibitory effect of

stilbene disulfonates (73–89%) is higher than in unmodified cells (65%), that of dipyridamole and salicylate are respectively comparable and somewhat lower, while inhibition by all other inhibitors is abolished or even turned into stimulation (Table 6). This lack of effect of pyridoxal phosphate and niflumate is in line with the disappearance of the inhibitory effect of pyridoxal phosphate on phosphate transport [29] and the loss of high affinity binding of flufenamate, an inhibitor structurally related to niflumate, to band 3 [39] after papain modification of erythrocytes.

After a sequential modification of cells by WRK/BH_4^- and papain ($k_1 = 5\ h^{-1}$), stilbene disulfonates maintain a high inhibitory potency (82–84%), while tetrathionate and dipyridamole exhibit respectively the high and low effectiveness characteristic for WRK/BH_4^- and other inhibitors exhibit the low effectiveness characteristic for the papain-treated cells (Table 6).

3.8. Effects of DIDS, papain and WRK / BH_4^- on ATP-independent outward translocation (flop)

After blockage of the ATP-dependent flop by a pretreatment of cells with *N*-ethylmaleimide or phenylarsine oxide (resp. 0.8 and 0.1 mmol l^{-1}, 15 min, 37°C), which decreases the rate constant of NBD-PA flop from 1.44 h^{-1} to 0.24 h^{-1} (Table 2), an involvement of band 3 in the residual flop is demonstrated by its inhibition by DIDS. The extent of inhibition varied from 16 to 34% ($n = 8$). Moreover, the DIDS-sensitive flop is stimulated by both papain (2.9-fold) and WRK/BH_4^- (1.9-fold), as found for the DIDS-sensitive flip. However, the extent of inhibition by DIDS and of stimulation by papain and WRK/BH_4^- are smaller than those found for the flip of NBD-PA (Tables 3 and 4). The variable DIDS sensitivity of the flop of NBD-PA remaining after blockage of the ATP-dependent process is probably due to an incomplete inhibition of this latter process, which is 7-fold faster than the flip. Such an incomplete suppression of the ATP-dependent flop may be derived from the 1.3-fold higher rate of the residual flop of NBD-PA ($k = 0.24 \pm 0.06\ h^{-1}$, Table 2) than that of the flip ($k = 0.19 \pm 0.02\ h^{-1}$, Table 1) in ATP-depleted cells and will reduce the apparent inhibitory effect of DIDS. Therefore, the flop remaining after suppression of both ATP-dependent and DIDS-sensitive flop ($k = 0.19 \pm 0.06\ h^{-1}$, $n = 6$) probably comprises in addition to a nonmediated component a residual ATP-dependent component.

4. Discussion

In the present model study, the inward and outward translocations of mono- and di-anionic NBD-labeled phospholipids have been found to depend on the structure and valence of the anionic phospholipid. The translocation pathways for these anionic NBD-labeled phospholipids

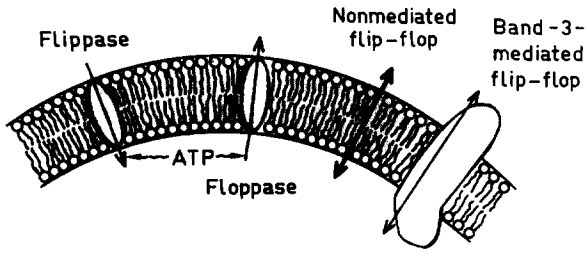

Fig. 8. Translocation pathways for anionic NBD-phospholipids in the erythrocyte membrane.

comprise two pathways for the inward translocation, one via the anion exchanger (AE1), band 3 protein, and one via a nonmediated process, as well as three pathways for the outward translocation, one via an ATP-dependent 'flippase', a second one via band 3 and a third one via a nonmediated process (Fig. 8).

The major route for the inward translocation of dianionic NBD-phospholipids is via band 3, whereas the major route for the inward translocation of monoanionic phospholipids is via a nonmediated process. In case of monoanionic NBD-phospholipids containing as a head group phosphate esters with apolar butyl and benzyl residues, the flip is a mere nonmediated process and a band-3-mediated component of flip is absent.

An involvement of band 3 in the flip of the dianionic NBD-phospholipids is supported by its suppression by DIDS (Table 3) and other well-established inhibitors of the anion exchanger. The inhibitory effect of a certain inhibitor of the anion exchange system on the flip of anionic NBD-phospholipids is influenced by both alterations of the structure of band 3, obtained by its modification by WRK/BH_4^- or papain, and the size as well as valence of the anionic head group of the phospholipid. Therefore, a lack of inhibition of flip by a given inhibitor of anion exchange does not necessarily mean that the inhibitor-insensitive flip is not mediated by band 3. The most potent inhibitor of flip is DIDS. It seems safe to conclude that the DIDS-sensitive component of anionic phospholipid flip is mediated by band 3.

Besides its physiological function of exchanging chloride against bicarbonate, the band 3 protein can accommodate a large variety of inorganic hydrophilic and organic monovalent as well as divalent anions [18,21,23,41–47]. The anion exchange protein has access channels on both the exofacial and endofacial sides of the protein separated by a thin permeability barrier [25,40,48,49]. A glutamate residue at position 681 in the band 3 protein is probably at the barrier and involved in H^+ binding during cotransport of H^+ and divalent anions [26]. Transformation of this negative glutamate residue in the band 3 protein into an alcohol by WRK/$NaBH_4^-$ inhibits transport of monovalent anions and stimulates that of divalent anions like sulfate [26] and amphilic NPP (data not shown). In line with this property of band-3-mediated transport, WRK/BH_4^- stimulates the flip of dianionic NBD-

phospholipids and abolishes the steep pH dependence of this flip (Fig. 6). From the model for the permeability barrier in the band 3 protein [40,48,49], the enhancement of the flip of dianionic NBD-phospholipids by WRK/BH_4^- would indicate that the rate-limiting step in this process is the diffusion of the polar head group of the dianionic NBD-phospholipds across the barrier rather than its diffusion to the barrier. An enhancement of NBD-PA flip by WRK/BH_4^- is still present following acceleration of flip by papain (data not shown), which cleaves two exofacial domains of the band 3 protein [27] between transmembrane segment 5 containing the exofacial DIDS reaction site (Lys 539) and segment 8 containing Glu 681. This shows that the stimulation of flip by papain is not the result of an enhanced diffusion of the dianionic head group of the phospholipid to the translocation site, but has to be due to a facilitation of its diffusion across the permeability barrier. Papain may be postulated to facilitate the conformational change of band 3 required for translocation of the anionic phospholipid.

Although a small to moderate suppression of translocation of monoanionic NBD-phospholipids by DIDS indicates an involvement of band 3 in this process, the major component of this translocation is probably nonmediated. Firstly, noncovalent inhibitors of anion exchange have no effect on the translocation of the monoanionic NBD-phospholipids, whereas they inhibit translocation of monoanionic ^{14}C-LPM [8] and DENSA [9]. Phloretin even stimulates translocation of monoanionic NBD-phospholipids. Secondly, in contrast to the lack of an inhibitory effect on the translocation of the monoanionic diacyl NBD-phospholipids (Table 4), WRK/BH_4^- has been found to inhibit the translocation of monoanionic ^{14}C-LPM [8] and DENSA [9]. Such inhibitory effects have to be expected from the reported inhibitory effect of WRK/BH_4^- on the band-3-mediated transport of chloride [25]. Thirdly, contrary to the DIDS-sensitive translocation of NBD-PA and DENSA, the translocations of the monoanionic NBD-phospholipids are not sensitive to changes of the anionic milieu (data not shown). Fourthly, the stimulating effect of papain on the translocation of the monoanionic phospholipids is considerably smaller than that on the band-3-mediated translocation of the monoanionic membrane-inserted amphiphiles ^{14}C-LPM [8] and DENSA [9]. Taken together, these results can be taken as evidence against a major role of band 3 in translocation of the monoanionic NBD-phospholipids.

The mechanism of flip of monoanionic NBD-phospholipids and dianionic NBD-PA via the DIDS-insensitive pathway is unclear. From the small pH dependence of the flip of monoanionic NBD-phospholipids, it can be concluded that its mechanism differs from that of the non-ionic diffusion-like process described for pure lipid bilayers. In lipid vesicles translocations of PG and PA increase 10-fold upon a decrease of pH by one unit [50,51]. The DIDS-insensitive flip is therefore proposed to represent the movement of the ionized phospholipids and to take place at structural packing defects in the hydrophobic barrier [1,2]. In line with this idea, the rate constants for the DIDS-insensitive flip of NBD-labeled PA and PG (about 0.05 and 0.03 h^{-1} at pH = 7.4; Tables 1 and 3) are comparable with the rate constant for the nonmediated flip of NBD-PC (0.02 h^{-1}, Table 1). Moreover, the rate constants for the DIDS-insensitive flip of NBD-labeled PGc and PG containing as a head group phosphate esters of hydrophilic residues are two orders of magnitude smaller than those for the flip of NBD-labeled PM, PB, and PBz containing as a head group phosphate esters of hydrophobic methyl, butyl and benzyl residues.

The major component of the outward translocation of anionic NBD-phospholipids represents a fast, mediated and ATP-dependent process (Table 2), which is inhibitable by vanadate, fluoride, SH-reagents or Mg^{2+}-depletion of cells. In contrast to the ATP-dependent aminophospholipid flippase [12], the ATP-dependent outward translocation system (floppase) exhibits only moderate substrate specificity, since it accepts zwitterionic, monoanionic and dianionic NBD-phospholipids (Table 2) as well as NBD-labeled PS [7] carrying two negative charges and one positive charge. Moreover, the outward translocation of spin-labeled PC, PE and PS has also been reported to be an Mg^{2+}-dependent process with rate constants of 0.18, 0.54 and 0.72 h^{-1} [6] comparable to the rate constants for the flop of NBD-labeled PC, PE and PS (0.76, 0.44 and 0.47 h^{-1}, Table 2). Previously, we found evidence for a protein-mediated outward translocation for PC synthesized by acylation of lysoPC at the inner surface of the erythrocyte membrane [5]. In contrast to the flop of NBD-PC, the flop of newly synthesized PC was not inhibitable by vanadate [5]. Moreover, the activation energy for the flop of NBD-PC is considerably higher (104 kJ/mol between 10 and 30°C, data not shown) than that for the outward translocation of newly synthesized PC (30 kJ/mol, Ref. [5]). Therefore, both processes are unlikely to be related. The erythrocyte membrane also contains an ATP-dependent, vanadate-inhibitable, transport system for the export of glutathione S-conjugates and organic anions [52,53]. A possible relationship between this pump and the outward translocation of NBD-phospolipids has to be investigated.

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