Band 3, an Accidental Flippase for Anionic Phospholipids? †

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Received October 12, 1993; Revised Manuscript Received January 11, 1994®

ABSTRACT: The inward translocation of the monovalent anionic phospholipid 1-palmitoyl-*sn*-glycero-3-phosphomethanol in the membrane of human erythrocytes is a fast process ($t/2$ = 11 min, 37 °C). Translocation of the protonated uncharged phospholipid is not responsible for the fast flip rate, and mediation of translocation by the aminophospholipid flippase could be excluded. Involvement of the anion exchanger band 3 in this process was derived from its inhibition (40–70%) by several established inhibitors of band 3-mediated anion exchange and its acceleration after proteolysis of band 3 by external papain. The translocation of the dianionic NBD-labeled phosphatidic acid is 5-fold slower, but also affected by the inhibitors. Thus, the anion exchanger can act as a flippase, defined as a transporter accepting substrates from the lipid bilayer.

The membrane of human erythrocytes contains an aminophospholipid flippase mediating fast ATP-dependent inward translocation of the phospholipid species characteristic for the inner membrane leaflet, phosphatidylserine and phosphatidylethanolamine (Devaux, 1991). On the other hand, inward translocation of the phospholipid species of the outer membrane leaflet, phosphatidylcholine and sphingomyelin, and of lysophospholipids with different structures for their polar head group is nonmediated and slow or even essentially absent (Middelkoop et al., 1986; Haest et al., 1986; Devaux, 1991). Interestingly, protein-mediated outward translocation of phosphatidylcholine species (Andrick et al., 1991; Connor et al., 1992) and inner leaflet phospholipid species (Bitbol & Devaux, 1988; Connor et al., 1992) has also been described. In the course of studies aiming at characterization of possible pathways of such flip processes, we have now obtained evidence that a "normal" transporter involved in anion exchange mediates the transbilayer movement of two simple anionic phospholipids, 1-(1-[^{14}C]palmitoyl)-*sn*-glycero-3-phosphomethanol (^{14}C-LPM)[1] and 1-oleoyl-2-[[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoyl]-*sn*-glycero-3-phosphate (NBD-PA).[1]

EXPERIMENTAL PROCEDURES

Materials. Erythrocytes were isolated from human blood. Papain, phospholipase D, niflumic acid, and dipyridamole were obtained from Sigma, 2,4,6-trinitrobenzenesulfonic acid (TNBS) was from ICN Biomedicals, phloretin was from Roth (Karlsruhe), pyridoxal 5′-phosphate was from Serva, Woodward's reagent K (WWK) was from Fluka, albumin was from Paesel (Frankfurt), 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) was from Calbiochem, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonic acid (H_2DIDS) was from HSC (Toronto, Canada), and 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS) was from Pfaltz and Bauer (Waterbury, CT). Incubation media contained (mmol·L^{-1}) KCl (90), NaCl (45), sucrose (44), and phosphate (12.5) or Hepes buffer (10), termed KNPS and KNHS.

Synthesis of Phospholipid Probes. LPM and NBD-PA were synthesized from 1-(1-[^{14}C]palmitoyl)-*sn*-glycero-3-phosphocholine (^{14}C-LPC; Dupont NEN) and 1-oleoyl-2-[[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoyl]-*sn*-glycerol-3-phosphocholine (NBD-PC; Avanti Polar Lipids), respectively, by enzymatic transphosphatidylation using phospholipase D from *Streptomyces* species according to Juneja et al. (1988). Briefly, 0.2 μmol of ^{14}C-LPC was dissolved in 200 μL of ethyl acetate and 100 μL of a solution containing 0.1 mol·L^{-1} MES, 0.1 mol·L^{-1} $CaCl_2$, and 3.2 mol·L^{-1} methanol. After addition of 2.5 units of phospholipase D, the mixture was shaken for 30 min at 37 °C. To stop the reaction, 120 μL of 0.25 mol·L^{-1} EGTA was added. The organic upper phase was then removed under a stream of N_2, and 60 μL of 0.02 N HCl was added (pH 3–4). Subsequently, lipids were extracted with 1 mL of methylene chloride/methanol (1:1) followed by 1 mL of methylene chloride. The combined extracts were washed once with 400 μL of 0.02 N HCl. Synthesis of NBD-PA was achieved by the same procedure starting from 10 μmol of NBD-PC except that all volumes were doubled, methanol was omitted in the reaction buffer, and lipid extraction was done with three portions of 1 mL of methylene chloride. The lipid products were purified by thin-layer chromatography on silica plates impregnated with 2.3% boric acid in ethanol using chloroform/ethanol/triethylamine/water (25:35:6:35) (Leray Pelletier, 1987). The ^{14}C-labeled lipids were detected by their radioactivity using a Berthold TLC linear analyzer LB 283 (Wildbad) and the fluorescent lipids by their yellow color or fluorescence. The products were identified by comparison of their R_f values with those of appropriate reference phospholipids. In the solvent system used, LPM is well separated from the starting phospholipid LPC and the only possible byproduct LPA (R_f values of 0.68, 0.13, and 0.45). The yield of LPM was 66 ± 10%. The solvent system also gives a good separation between the single product NBD-PA and the

† This work was supported by the Deutsche Forschungsgemeinschaft (Ha 154/1-2).

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® Abstract published in *Advance ACS Abstracts,* March 15, 1994.

[1] Abbreviations: LPC, 1-palmitoyl-*sn*-glycero-3-phosphocholine; LPM, 1-palmitoyl-*sn*-glycero-3-phosphomethanol; NBD-PA, 1-oleoyl-2-[[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoyl]-*sn*-glycero-3-phosphate; NBD-PC, 1-oleoyl-2-[[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-amino]hexanoyl]-*sn*-glycero-3-phosphocholine; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonic acid; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonic acid; TNBS, 2,4,6-trinitrobenzenesulfonic acid; WWK, Woodward's reagent K [2-ethyl-5-(3-sulfophenyl)isoxazolium].

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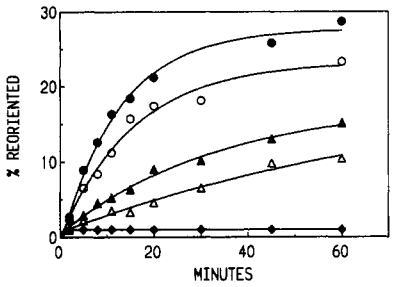

FIGURE 1: Time-dependent reorientation of LPM from the outer to the inner membrane leaflet of human erythrocytes at different temperatures. ^{14}C-Labeled LPM was inserted into the outer membrane leaflet of human erythrocytes (4 °C), and its translocation to the inner leaflet was measured (37 °C, KNPS, pH 7.4) by following the time-dependent decrease of extractability of radioactivity by albumin (Schwichtenhövel et al., 1992) at 4 (♦), 25 (△), 30 (▲), 37 (○), and 41 (●) °C.

starting fluorescent phospholipid NBD-PC (R_f values of 0.51 and 0.10).

Modification of Erythrocytes. Erythrocytes were pretreated with DIDS or H_2DIDS (30 min, 37 °C, KNPS, pH 7.4), TNBS (30 min, 37 °C, KNPS, pH 8.0), or WWK followed by $NaBH_4$ (both 10 min, 0 °C, KNHS, pH 7.0) and washed 3 times with medium. For proteolytic digestion of band 3, erythrocytes in KNPS (50% hematocrit) were treated with cysteine-activated papain (30 units/mL of cells, 60 min, 37 °C, pH 7.4) and washed twice with 0.2% albumin in KNPS and 2 times with KNPS.

Flip Measurements. Following insertion of trace amounts of ^{14}C-LPM (13 nmol/mL of cells) or NBD-PA into the outer membrane leaflet of erythrocytes (10 min, 4 °C), the rates of inward translocation were derived from the time-dependent increase of radioactivity or fluorescence not extractable by albumin (Schwichtenhövel et al., 1992). The stability of the probes during flip was checked by TLC.

RESULTS AND DISCUSSION

Figure 1 shows the time-dependent transmembrane inward reorientation of LPM at different temperatures. By fitting the data to an exponential using least-squares, nonlinear regression, a flip rate constant of 0.93 ± 0.16 h^{-1} (n = 54, $t/2$ = 11 min, 37 °C, pH 7.4) was obtained. This flip rate of the anionic LPM is 50-fold higher than that of its zwitterionic analogue, palmitoyllysophosphatidylcholine (k = 0.019 h^{-1}; Haest et al., 1986). From the Arrhenius plot, an activation energy of 79 ± 8 kJ·mol^{-1} was derived. Similar high activation energies have been reported for the nonmediated flip of lysophospholipids and phosphatidylcholine in erythrocytes (Haest et al., 1986; Zachowski et al., 1986) and of various diacylphospholipids in lipid vesicles (Homan & Pownall, 1988). In the range between 5 and 100 nmol of LPM/mL of cells (0.001–0.025 mol of LPM/mol of membrane phospholipid), the amount of LPM translocated per minute increases linearly with the amount inserted into the membrane (data not shown).

The rate of LPM translocation is considerably higher than that of the nonmediated flip of other phospholipids and lysophospholipids in erythrocytes ($t/2$ = 200–1500 min: Middelkoop et al., 1986; Haest et al., 1986). It is comparable to the rates of the flippase-mediated translocation of the aminophospholipids phosphatidylserine and phosphatidylethanolamine ($t/2$ = 5 and 60 min; Devaux, 1991). An involvement of this flippase in the flip of LPM could, however, be excluded, since inhibition of the flippase activity (Devaux, 1991) by ATP depletion of the cells, by pretreatment of the cells with *N*-ethylmaleimide, or by loading cells with vanadate or Ca^{2+} did not inhibit the flip of LPM (data not shown).

Table 1: Suppression of the Flip of LPM and NBD-PA by Inhibitors of Anion Exchange[a]

inhibitor	concentration (mmol·L^{-1})	% inhibition LPM	% inhibition NBD-PA
DIDS	0.005	38	
	0.050	54 ± 12	65
H_2DIDS	0.010		66 ± 7
TNBS	5.0	67 ± 8	
WWK/$NaBH_4$	1.8	40	0[b]
DNDS	0.500	46 ± 14	63
dipyridamole	0.020		
in chloride		50 ± 14	47
in sulfate		0–15	
pyridoxal-5-P	15.0	61	
	30.0	71	45
phloretin	0.25	37	
	0.60	44 ± 6	0
niflumic acid	0.020	0	35
tetrathionate	25.0		52
salicylate	40.0		34

[a] Erythrocytes were pretreated with DIDS, H_2DIDS, TNBS, or WWK/$NaBH_4$ (see Experimental Procedures). Probes were inserted into the outer membrane leaflet (4 °C), and inward flip was measured (37 °C, KNPS, pH 7.4). To study the effects of noncovalent inhibitors, the flip was measured in the absence and presence of DNDS, phloretin, niflumic acid, salicylate, tetrathionate, pyridoxal 5-phosphate, or dipyridamole. Inhibition data represent mean values from 2–27 experiments. SD values are given in the case of at least four experiments. [b] No inhibition, but acceleration (see text).

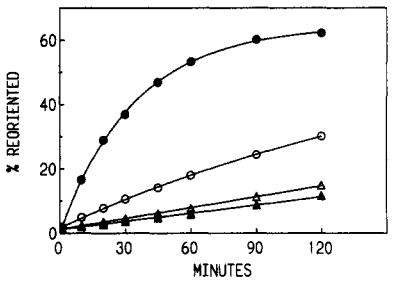

FIGURE 2: Effects of H_2DIDS and WWK/$NaBH_4$ on the flip of NBD-PA. NBD-PA was inserted into membranes of freshly washed erythrocytes or of cells pretreated (0 °C) with WWK/$NaBH_4$ (see Experimental Procedures), and flip was measured in the absence or presence of DIDS (50 μmol·L^{-1}). No pretreatment: flip without (○) and with (△) DIDS. WWK/$NaBH_4$: flip without (●) and with (▲) DIDS.

Moreover, transbilayer movement of the protonated uncharged form of LPM, equivalent to nonionic diffusion, cannot explain the high flip rate. First, the pK value for the dissociation of the phosphate group of membrane-inserted LPM is about 2 (Tocanne & Teissié, 1990). Second, the flip rate only changes by a factor of 1.3 per pH unit between pH 6 and 9.5 (data not shown), whereas a 10-fold change would be expected in the case of a translocation of LPM as the protonated uncharged acid.

Evidence for the involvement of a protein in the flip of the amphiphilic anion LPM comes from experiments with inhibitors of anion exchange via the band 3 protein. At concentrations sufficient to fully suppress anion exchange (Cabantchik & Greger, 1992), DIDS was found to inhibit the flip of LPM to a maximum of 54 ± 12% (Table 1).

The flip of another anionic phospholipid, NBD-PA, also proved to be inhibited by DIDS and other stilbenedisulfonates (Figure 2). The maximal inhibition by H_2DIDS was 66 ± 7% (Table 1) and the half-maximal concentration (I_{50}) about 1.5 μmol·L^{-1}. NBD-PA, a predominantly divalent anion at pH

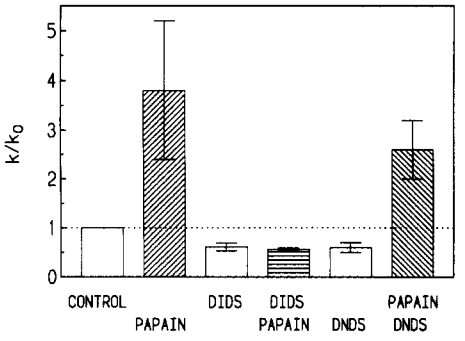

FIGURE 3: Acceleration of flip by proteolytic cleavage of band 3 by external papain. Freshly washed erythrocytes or cells pretreated with DIDS (50 μmol·L^{-1}, 30 min, pH 7.4) were exposed to papain and washed (see Experimental Procedures). LPM was then inserted into the membrane and its inward flip measured in the absence or presence of DNDS. Mean ± SD, n = 3–4.

7.4 (pK_{a_2} about 6.3; Tocanne & Teissié, 1990), undergoes inward flip at a rate of 0.18 ± 0.02 h^{-1} (n = 14; $t/2$ = 120 min) with a high activation energy of 110 kJ·mol^{-1}. This flip rate is about 5 times slower than that of monovalent LPM, but 10-fold faster than that of zwitterionic NBD-phosphatidylcholine (Schwichtenhövel et al., 1992). Moreover, the flip of NBD-PA does not exhibit the strong pH dependence (10-fold per pH unit) of the translocation of PA in pure lipid vesicles (Eastman et al., 1991), indicating the involvement of "nonionic diffusion". In erythrocytes, the flip rate of NBD-PA increase only 3.4-fold upon a decrease of the pH from 8.9 to 6.5 (data not shown).

Inhibition of the flip of LPM and NBD-PA in the range of 35–70% was also observed for other established (Cabantchik & Greger, 1992; Renner et al., 1988; Cabantchik et al., 1975; Deuticke, 1970; Knauf & Rothstein, 1971) inhibitors of anion exchange via band 3, such as DNDS, dipyridamole, salicylate, tetrathionate, or pyridoxal 5′-phosphate, present during flip and after irreversible modification of cells with TNBS. Interestingly, the flip of LPM is suppressed (44%) in the presence of the inhibitor phloretin (Fröhlich & Gunn, 1987), whereas the flip of NBD-PA is not (Table 1). For another inhibitor of anion exchange, niflumic acid (Cousin & Motais, 1979), the reverse situation is observed: the flip of NBD-PA is inhibited (35%) and that of LPM is not (Table 1). Although no explanation for the opposite effects of niflumic acid and phloretin on the flip of LPM and NBD-PA can be given, it may be relevant to mention that both noncompetitive inhibitors of anion exchange do not inhibit the slow net anion flow via band 3 (Fröhlich, 1988; Knauf, 1989).

Three further findings support a role for band 3 in the flip of LPM and NBD-PA.

(1) As a common feature of the inward translocation of LPM and of anion transport (Renner et al., 1988), their inhibition by dipyridamole depends on the presence of Cl$^-$. Replacement of intra- and extracellular Cl$^-$ by sulfate removes the inhibitory effect of dipyridamole on the flip of LPM (Table 1).

(2) Treatment of erythrocytes with papain, which cleaves band 3 into two large and two small peptides (Jennings & Adams, 1981; Jennings et al., 1984), accelerates the flip of LPM about 4-fold (Figure 3) and that of NBD-PA 6-fold (data not shown). Papain treatment has been reported to accelerate the inward transport of anions via band 3 under influx-limited conditions (Jennings & Adams, 1981). The concentration and pH dependence of the papain effect on flip acceleration correlate with the extent of band 3 cleavage (data not shown). Treatment of cells with DIDS prior to papain treatment, known to interfere with band 3 cleavage (Jennings et al., 1984), prevents the flip acceleration (Figure 3). Moreover, the inhibition of LPM flip by DNDS is less pronounced in papain-treated cells (Figure 3), analogous to anion transport (Jennings & Adams, 1981; Matsuyama et al., 1983). Treatment of erythrocytes with trypsin or chymotrypsin was not effective.

(3) Analogous to findings for the transport of small polar anions (Jennings & Al-Rhaiyel, 1988), the flip of a monoanionic phospholipid, LPM, is inhibited by sequential treatment of cells with WWK and $NaBH_4$ (Table 1), whereas that of a dianionic phospholipid, NBD-PA, is accelerated up to 7-fold at pH 7.4 (Figure 2). The WWK-induced increment of flip of NBD-PA is completely suppressed by DIDS (Figure 2). Sequential treatment of cells with papain and WWK/$NaBH_4$ even stimulates the flip of NBD-PA about 25-fold ($t/2$ = 5 min). In contrast, the nonmediated flip of NBD-PC and the aminophospholipid translocase-mediated flip of NBD-PS are not affected by such a sequential treatment (data not shown).

All these results suggest that the anion exchanger band 3, besides its normal function of transporting a wide variety of hydrophilic anions (Cabantchik & Greger, 1992), including phosphate (Deuticke, 1970), phosphate analogs (Labotka & Omachi, 1987), and phosphate esters (Cabantchik et al., 1975; Hamasaki et al., 1978), from the *cis* to the *trans* aqueous compartment of the membrane, can also mediate the transbilayer reorientation of long-chain amphiphilic anionic substrates already present in the lipid domain of the membrane after a preceding step of partitioning (measured membrane/water partition coefficient of LPM >5 × 10^5).

Assuming that the transfer sites of the obligatory exchange system for "normal" anions are also involved in the translocation of the anionic phospholipid head group across the membrane barrier, the incomplete inhibition of flip by blockers of anion exchange can be explained in two ways. On the one hand, one could postulate two parallel routes for the translocation: one mediated by band 3 and one nonmediated. In line with this idea, the DIDS-insensitive flip is accelerated by pretreatment with the SH oxidant diamide (data not shown), known to accelerate the nonmediated flip of lysophospholipids (Bergmann et al., 1984; Haest et al., 1986) and NBD-phosphatidylcholine (data not shown). In this case, the nonmediated flip of NBD-PA in erythrocytes would be different from the translocation of uncharged protonated PA described for pure lipid vesicles (Eastman et al., 1991). On the other hand, both the DIDS-sensitive flip and a major part of the DIDS-insensitive flip could be mediated by band 3. One would then have to claim different inhibitor profiles for phospholipid translocation and anion exchange.

Alternatively, the incomplete inhibition could be the consequence of a mechanism of phospholipid translocation different from that of electrically silent obligatory anion exchange, but related to the conductive mode of operation of band 3 (Fröhlich, 1988). In line with this idea, we found that the flips of LPM and NBD-PA are electrogenic processes (Haest & Jansen, 1993). As further evidence for a "nonclassical" operation of band 3 during translocation of the anionic phospholipids, butanol accelerates the DIDS-sensitive flip (Figure 4), while the exchange of hydrophilic anions is known to be inhibited (Forman et al., 1985). An alcohol-induced acceleration has already been reported both for the flippase-mediated (Bassé et al., 1992) and for nonmediated flip processes (Orme et al., 1988; Schwichtenhövel et al., 1992).

In summary, band 3, besides its physiological operation of transporting hydrophilic anions, thus provides, albeit only

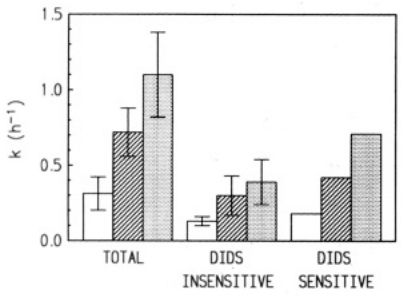

FIGURE 4: Effect of butanol on the total, the DIDS-insensitive, and the DIDS-sensitive flip of LPM. Cells were treated with DIDS (50 μmol·L^{-1}, 37 °C, 30 min, pH 7.4) and washed. Flip was measured in the absence (empty bars) and presence of 75 (diagonally hatched bars) and 100 mmol·L^{-1} butanol (speckled bars) in control and DIDS-treated erythrocytes. The flip rate of the DIDS-sensitive component was calculated from the difference of flip rates in untreated and DIDS-treated cells. Mean ± SD, n = 3.

accidentally, a pathway for the simultaneous translocation of the hydrophilic head group and the hydrophobic acyl chain(s) of certain anionic phospholipids following their approach from the lipid domain. Proteins with such activities have been termed flippases (Devaux, 1992). The results presented here demonstrate that there may not necessarily be principal differences between such flippases and "classical" transporters.

ACKNOWLEDGMENT

We are indebted to Mrs. D. Kamp for her skillful technical assistance.

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