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Phospholipid Flop Induced by Transmembrane Peptides in Model Membranes Is Modulated by Lipid Composition

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ABSTRACT: Since phospholipid synthesis is generally confined to one leaflet of a membrane, membrane growth requires phospholipid translocation (flip-flop). It is generally assumed that this process is protein-mediated; however, the mechanism of flip-flop remains elusive. Previously, we have demonstrated flop of 2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl] (C_6NBD) phospholipids, induced by the presence of membrane-spanning peptides in vesicles composed of an *Escherichia coli* phospholipid extract, supporting the hypothesis that the presence of transmembrane stretches of proteins in the bilayer is sufficient to allow phospholipid flip-flop in the inner membrane of *E. coli* [Kol et al. (2001) *Biochemistry 40,* 10500]. Here, we investigated whether the specific phospholipid composition of *E. coli* is a prerequisite for transmembrane helix-induced flop of phospholipids. This was tested by determining the amount of C_6NBD-phospholipid that was translocated from the inner leaflet to the outer leaflet of a model membrane in time, using a dithionite reduction assay. The transmembrane peptides GWWL$(AL)_8$WWA (WALP23) and GKKL$(AL)_8$KKA (KALP23) induced phospholipid flop in model membranes composed of various lipid mixtures. The rate of peptide-induced flop was found to decrease with increasing dioleoylphosphatidylethanolamine (DOPE) content of vesicles composed of DOPE and dioleoylphosphatidylcholine (DOPC), and the rate of KALP23-induced flop was shown to be stimulated by higher dioleoylphosphatidylglycerol (DOPG) content in model membranes composed of DOPG and DOPC. Furthermore, the incorporation of cholesterol had an inhibitory effect on peptide-induced flop. Finally, flop efficiency was strongly dependent on the phospholipid headgroup of the NBD-phospholipid analogue. Possible implications for transmembrane helix-induced flop in biomembranes in general are discussed.

Membrane biogenesis is a process of fundamental importance in cell biology, because membrane expansion is a prerequisite for cellular growth. It requires the incorporation of lipids and proteins in the growing membrane. Glycerophospholipids are the most abundant membrane lipids in eukaryotes and most prokaryotes. The bulk of the membrane glycerophospholipids is synthesized in so-called biogenic membranes, the endoplasmic reticulum (ER)[1] membrane in eukaryotes and the cytoplasmic membrane in bacteria. From their site of synthesis, they reach their destination via inter- and intramembrane transport. Intermembrane transport of lipids is generally thought to proceed via membrane contact sites (*1*, *2*), and, in eukaryotes, by vesicular traffic (see, e.g., ref *3*). Intramembrane transport occurs either within one leaflet of the membrane (lateral diffusion) or from one membrane leaflet to the other. The latter, phospholipid flip-flop, is at the basis of membrane biogenesis since lipid synthesis is *asymmetric*, i.e., it is confined to one leaflet of the membrane, and thus efficient flip-flop of lipids is required to ensure balanced growth of the bilayer.

In vitro assays with labeled phospholipids have revealed several general characteristics of flip-flop in isolated membrane preparations of ER (microsomes) and bacterial cytoplasmic membranes (*4*−*9*): phospholipid translocation is fast ($t_{1/2}$ seconds to minutes), headgroup aspecific, and independent of an energy source or other cytosolic factors. Thus, phospholipid translocation appears to be an intrinsic property of biogenic membranes. In contrast, in model membranes composed of lipid extracts of biogenic membranes, flip-flop is slow ($t_{1/2}$ days to weeks). Therefore, it is generally accepted that phospholipid translocation is mediated by (a) membrane protein(s), and indeed in some studies a (partial) sensitivity toward proteases (*6*) and the alkylating agent *N*-ethylmaleimide (*4*) has been reported.

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[1] Abbreviations: C_6-NBD-PL, 1-palmitoyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]-*sn*-glycero-3-phospholipid; EDTA, ethylenedinitrilotetraacetic acid; ER, endoplasmic reticulum; IMV, inner membrane vesicle; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; PG, phosphatidylglycerol; PS, phosphatidylserine; PL, phospholipid(s); $t_{1/2}$, translocation half-time; TEA, triethanolamine; TLE, total lipid extract of *Escherichia coli*.

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Consistent with this view, phospholipid flip-flop has been observed in proteoliposomes, in which membrane protein extracts derived from rat liver microsomes (*10*, *11*), *Bacillus subtilis* IMV (*12*), or *Escherichia coli* IMV (*9*) were reconstituted. Flip-flop is bidirectional and partially protease sensitive. Fractionation of the protein extract and subsequent reconstitution result in proteoliposomes with varying flop activities, opening up possibilities for isolation and identification of the putative flippase(s).

Until now, the identities and mode of action of these flippases have remained elusive. The main translocation barrier for the polar phospholipid headgroups is the hydrophobic interior of the membrane. Thus, we have hypothesized that the transmembrane α-helices spanning the bilayer, a general motif in membrane proteins, facilitate phospholipid flip-flop. Previously (*13*), we found support for this hypothesis in a study on vesicles composed of an *E. coli* phospholipid extract containing α-helical transmembrane peptides with a Leu-(Ala-Leu)$_n$ hydrophobic core flanked on both sides by anchoring residues (see Experimental Procedures section), as a model for the *E. coli* inner membrane. The dithionite reduction assay was used to show that the peptides induced flop of short-chain NBD analogues of PE and PG, the predominant phospholipid classes found in the *E. coli* inner membrane (~80 and ~15 mol %, respectively).

To investigate whether the typical lipid composition of the *E. coli* inner membrane is required for transmembrane peptide-induced flop, we studied flop in peptide-containing model membranes with other lipid compositions. The peptides studied contained either Trp or Lys as flanking residues, because these amino acids are frequently found in membrane proteins at the membrane−water interface. We found that peptide-induced flop is not restricted to model membranes composed of *E. coli* phospholipids but occurs in vesicles of various lipid compositions. The rate of phospholipid translocation was modulated by the lipid composition of the membrane. The results provide further support for the hypothesis that flop of phospholipids is mediated by the membrane-spanning α-helical stretches of proteins and will be discussed in the light of possible implications for phospholipid translocation in biomembranes.

EXPERIMENTAL PROCEDURES

Materials. The *E. coli* total lipid extract was isolated from the wild-type strain W3899, which was grown to the late log phase. Cells were harvested and washed, and the lipids were extracted and subsequently purified on a silica column as described previously (*13*). All other lipids were obtained from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Stock solutions of all lipids were prepared in chloroform, stored under N_2 at −20 °C, and periodically checked by thin-layer chromatography. The peptides WALP23 [Ac-GWWL(AL)$_8$WWA-amide] and KALP23 [Ac-GKKL(AL)$_8$KKA-amide] were synthesized as described (*14*, *15*). Stock solutions of the peptides in trifluoroethanol (0.48 mM) were prepared on the basis of weight and stored under N_2 at −20 °C.

Preparation of Large Unilamellar Vesicles. Vesicles were prepared as described previously (*13*). Briefly, a lipid film was prepared, consisting of the phospholipids indicated, peptide at the appropriate molar ratio (up to a 1:100 peptide/phospholipid ratio), and an NBD-phospholipid analogue (0.2 mol % of total PL). The lipid film was hydrated to a final concentration of 5 mM phospholipid by adding buffer Z (10 mM KCl, 50 mM TEA, 1 mM EDTA, pH 7.5), supplemented with 20 mM $K_3Fe(CN)_6$, which was added to scavenge minor amounts of dithionite permeating into the vesicle lumen. After repetitive freezing and thawing and subsequent extrusion through 200 nm membrane filters (Anotop 10; Whatman, Maidstone ,U.K.), the unilamellar vesicles were separated from extravesicular $K_3Fe(CN)_6$ by gel filtration on a Sephadex G-50 spin column.

The symmetrically labeled vesicles were incubated with 25 mM sodium dithionite ($Na_2S_2O_4$) for 5 min to reduce and thereby quench the fluorescent NBD-label in the outer membrane leaflet, followed by gel filtration as above to remove excess dithionite. This procedure resulted in asymmetrically labeled vesicles, i.e., vesicles in which the probe is confined to the inner leaflet, that were immediately used in the flop assay. For the control experiments, vesicles without peptide were prepared following the same procedure. Sample preparation was performed at 25 °C.

Flop Assay. Flop was measured as described previously (*13*). The asymmetrically labeled vesicles were incubated at a concentration of ~3 mM PL-P_i in buffer Z at 25 °C. At different time points, 50 μL aliquots were transferred to 1150 μL of buffer Z in a cuvette in a spectrofluorometer. The amount of NBD-phospholipid reduced by 8 mM sodium dithionite in 3 min was taken as the amount of flop. Control experiments showed that addition of dithionite to an aliquot of symmetrically labeled vesicles resulted in an ~50% decrease in fluorescence intensity, irrespective of the lipid composition of the vesicle or the presence of peptide, indicating that vesicles with and without peptide are largely sealed to dithionite and unilamellar. Fluorescence measurements were performed in buffer Z using an SLM Aminco SPF 500C spectrofluorometer (excitation 460 nm, emission 534 nm). From these measurements the percentage of NBD-phospholipid in the outer leaflet was calculated according to

$$\text{flop (\%)} = [1 - (F_{180}/F_0)] \times 100 \qquad (1)$$

with F_0 and F_{180} the fluorescence intensities after 0 and 180 s of reaction with dithionite, respectively.

Equation 2 theoretically describes the process of flip-flop in a vesicle assuming that the inward and outward rate constants are equal (*16*):

$$\text{flop}(t) = 0.5(1 - e^{-K_{\text{flop}}t}) \qquad (2)$$

with the factor 0.5 representing the theoretical maximum value of flop (50% redistribution of the probe) and t the time of incubation (h).

In our system, flop at $t = 0$ variably deviated from the theoretical value of 0%. To be able to compare flop rate constants in vesicles of different lipid compositions, C_{offset} was introduced as a constant equal to the amount of accessible NBD label at $t = 0$ in control vesicles without peptide ($0.03 < C_{\text{offset}} < 0.1$, depending on the experiment). The apparent first-order flop rate constants (K_{flop}) were then calculated by a least-squares fit to the equation:

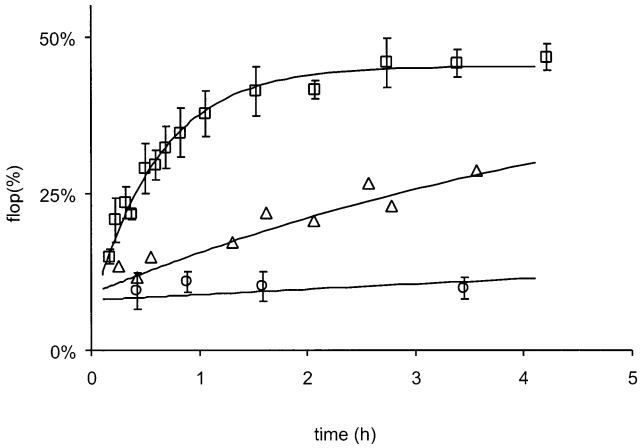

FIGURE 1: Time course of flop of C_6NBD-PG (0.2% of total phospholipid) in DOPC vesicles containing WALP23 [Ac-G**WW**L-(AL)$_8$**WW**A-amide] at a 1:1000 peptide/lipid (P/L) molar ratio (squares) and without peptide (circles). Data from several independent experiments were averaged (time points within ±3 min up to $t = 2$ h and ±20 min after $t = 2$ h) and used for least-squares curve fitting according to eq 3 (see Experimental Procedures section). For comparison, WALP23-induced flop of C_6NBD-PG in vesicles prepared of an *E. coli* lipid extract (1:1000 P/L, triangles, $n = 2$) is also shown.

$$\text{flop}(t) = C_{\text{offset}} + (0.5 - C_{\text{offset}})(1 - e^{-K_{\text{flop}}t}) \quad (3)$$

Miscellaneous. Phospholipids were quantified according to Rouser et al. (*17*). Phospholipid compositions and concentrations are presented on the basis of lipid phosphorus (PL-P_i).

RESULTS

In a previous study on transmembrane peptide-induced flop in model membranes composed of *E. coli* phospholipids, a time-dependent, peptide-mediated redistribution of fluorescent analogues of the predominant phospholipids in *E. coli*, C_6NBD-PE and C_6NBD-PG, was observed. Using the same approach, we tested whether transmembrane helix induced flop also occurs in other lipid systems.

In DOPC vesicles with the model peptide WALP23 incorporated at a 1:1000 molar ratio with respect to phospholipid, flop of C_6NBD-PG is observed (Figure 1, squares), with a translocation half-time of ~25 min, whereas in the absence of peptide (circles), flop is negligible as expected (*13*, *18*). This shows that transmembrane peptide-induced phospholipid flop is not restricted to model membranes with the natural phospholipid composition of *E. coli* but that it also occurs in vesicles composed of a synthetic phospholipid. Interestingly, WALP23-induced flop of C_6NBD-PG in TLE vesicles (1:1000 peptide/phospholipid, Figure 1, triangles) is much slower than in DOPC vesicles.

We further characterized WALP23-induced flop of C_6-NBD-PG in these two lipid systems by determining the apparent first-order rate constants (K_{flop}) of flop of C_6NBD-PG at different peptide/phospholipid molar ratios (P/L) (Figure 2). Both in TLE and in DOPC vesicles, the WALP23-induced flop rate increases linearly with the peptide concentration. However, the rate of WALP23-induced flop in TLE is 7-fold slower than in DOPC, as determined from the slopes of the two lines, demonstrating that the rate of WALP23-induced flop is strongly dependent on the lipid environment.

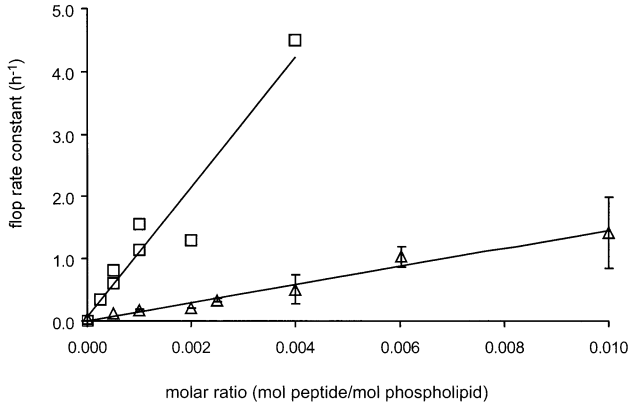

FIGURE 2: Apparent first-order flop rate constants (K_{flop}) of C_6-NBD-PG-flop, induced by WALP23, plotted against the peptide/phospholipid molar ratio in vesicles prepared of DOPC (squares) and of an *E. coli* lipid extract (TLE) (triangles). K_{flop} values were calculated according to eq 3 using least-squares analysis. For DOPC, individual data points, and for TLE, averaged data points ($n = 2$, no error bar, or $n = 3$, ±SD) are presented.

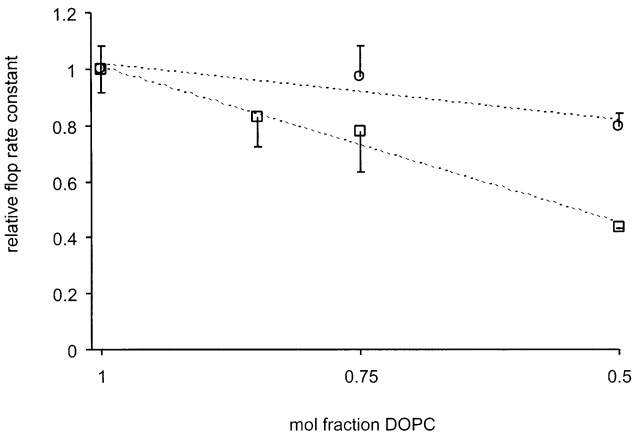

FIGURE 3: Dependence of K_{flop} of WALP23-induced flop of C_6-NBD-PG on the lipid composition of the vesicles. Flop rate constants were determined for vesicles containing DOPC and DOPG (circles) or DOPC and DOPE (squares) in various molar ratios as indicated on the *x*-axis and peptide (P/L = 1:1000). Data were normalized to K_{flop} determined in 100% DOPC. Averaged data points ($n = 3$, ±SD) are connected with dashed lines to guide the eye. For clarity, the upper (squares) and lower (circles) error bars have been omitted.

An *E. coli* phospholipid extract consists mainly of the zwitterionic, nonbilayer-preferring lipid PE (~80%) and the anionic PG (~15%). To investigate the influence of PE and PG on WALP-induced flop separately, vesicles were prepared composed of the bilayer-preferring lipid DOPC and increasing amounts of either DOPE or DOPG. Since a high DOPE content will destabilize the bilayer, molar fractions up to 0.5 were tested. The K_{flop} of WALP23-induced flop of C_6NBD-PG is decreased with increasing mole fractions of both DOPG (Figure 3, circles) and DOPE (squares), the latter showing the strongest effect. These results demonstrate that the rate of WALP23-induced flop is reduced by the two most abundant phospholipid classes found in *E. coli* and provide an explanation for the slower flop induced by WALP23 in vesicles composed of TLE, as compared to DOPC.

The efficient flop of C_6NBD-PG in WALP23-containing DOPC vesicles was used as a frame of reference to study flop of other phospholipid classes. Figure 4 shows the WALP23-induced flop of C_6NBD-PA, C_6NBD-PE, C_6NBD-

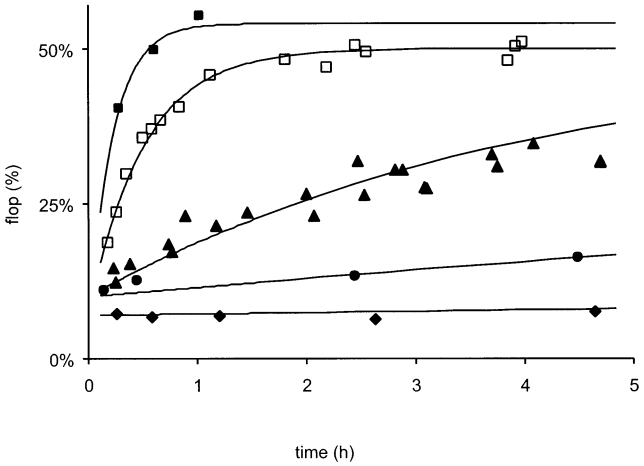

FIGURE 4: Time course of WALP23-induced flop of several C_6-NBD-phospholipids (0.2% of total phospholipid) in DOPC vesicles, C_6NBD-PA (squares), C_6NBD-PE (triangles), C_6NBD-PC (diamonds), and C_6NBD-PS (circles), at a peptide/PL molar ratio of 1:250 (solid symbols) and 1:1000 (open symbols). Data from a single experiment (C_6NBD-PS, C_6NBD-PC, and C_6NBD-PA, WALP23/PL 1:250) and from at least two independent experiments (other two data sets) are connected by a curve fitted according to eq 3 using least-squares analysis.

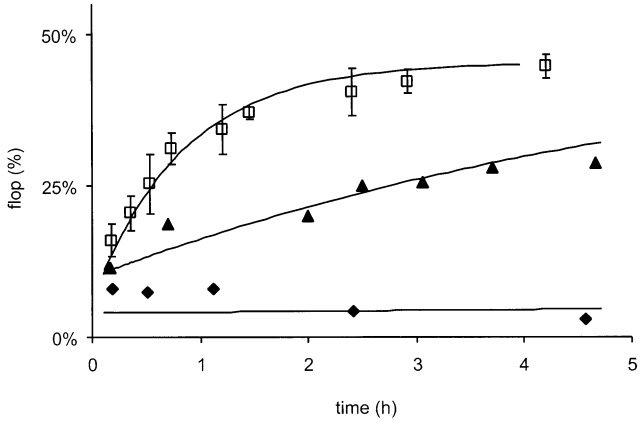

FIGURE 5: Time course of flop induced by KALP23 [Ac-G**KK**L-(AL)$_8$**KK**A-amide] of several C_6NBD-phospholipids (0.2% of total phospholipid) in DOPC vesicles, C_6NBD-PG (squares), C_6NBD-PE (triangles), and C_6NBD-PC (diamonds), at a peptide/PL molar ratio of 1:250 (solid symbols) or 1:1000 (open symbols). The data points are connected by curves fitted according to eq 3 using least-squares analysis. For C_6NBD-PG, data points (taken within 3 min up to 2 h of incubation and within 20 min at later time points) from several independent experiments are averaged and the SD ($n = 5$) indicated. For the other probes, data from single experiments are presented.

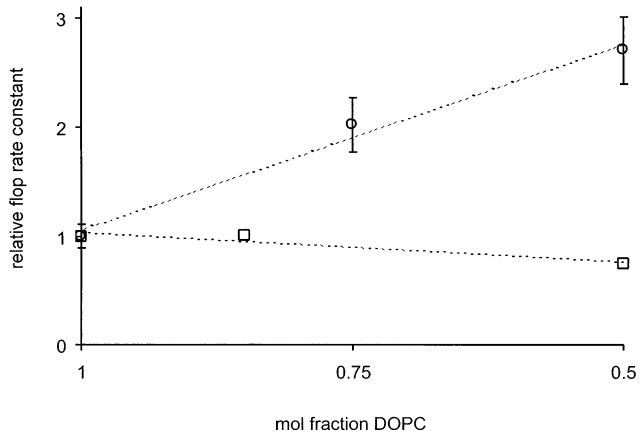

FIGURE 6: Dependence of K_{flop} of KALP23-induced flop of C_6-NBD-PG on the lipid composition of the vesicles. Flop rate constants were determined in vesicles containing DOPC and DOPG (circles) or DOPC and DOPE (squares) in various molar ratios as indicated on the *x*-axis and peptide (P/L = 1:1000). Data were normalized to the rate constant determined in 100% DOPC. Averaged data points (circles, $n = 3 \pm$ SD, and squares, $n = 2$) are connected to enhance the visual stimulus.

PC, and C_6NBD-PS, analogues of natural phospholipids that are widely distributed throughout biomembranes. The negatively charged C_6NBD-PA flops rapidly at a peptide/phospholipid molar ratio of 1:1000 (open squares), with a flop rate comparable to that of C_6NBD-PG ($t_{1/2}$ ~21 and ~25 min, respectively). WALP23-induced flop of C_6NBD-PA at a peptide/phospholipid ratio of 1:250 (solid squares) proceeded very rapidly. At this peptide concentration, the zwitterionic C_6NBD-PE (triangles) is also redistributed, albeit at a slower rate. Interestingly, neither the zwitterionic C_6-NBD-PC (diamonds) nor the negatively charged C_6NBD-PS (circles) display appreciable WALP-induced flop on this time scale, even when the peptide:phospholipid molar ratio was increased to 1:100 (not shown). None of the probes translocated in the absence of peptide (not shown). The data demonstrate that the rate of flip-flop depends not only on the bulk lipid composition of the membrane but also strongly on the nature of the translocating species.

We next tested phospholipid flop in DOPC vesicles induced by another peptide, KALP23, which contains four lysines as anchoring residues. Previously, we reported that this peptide is more efficient in inducing flop in TLE vesicles, as compared to the Trp-flanked WALP23 (*13*). Flop of C_6-NBD-PG was fast ($t_{1/2}$ of ~34 min) at a KALP23/phospholipid molar ratio of 1:1000 (Figure 5, open squares) and increased further at higher KALP23 concentrations (not shown). C_6NBD-PE (solid triangles, 1:250 P/L) displays a gradual KALP23-mediated redistribution in time. At this concentration, KALP23 does not induce measurable flop of C_6NBD-PC (solid diamonds), nor could flop of C_6NBD-PC be observed on this time scale at a 1:100 peptide/phospholipid molar ratio (not shown). Interestingly, whereas in TLE vesicles the rate of KALP23-induced flop of C_6NBD-PG was about a factor of 5 higher than that of WALP23-induced flop of C_6NBD-PG (*13*), in DOPC vesicles the rates of flop induced by WALP23 and KALP23 are comparable ($t_{1/2}$ of 25 and 34 min, respectively), indicating that changing the lipid composition of the model membrane has a differential effect on these two peptides.

To get insight into the roles of PG and PE in KALP23-mediated flop of C_6NBD-PG, phospholipid flop was measured in KALP23-containing model membranes composed of either DOPE or DOPG mixed with DOPC. Increasing the PE/PC ratio slightly reduced the relative flop rate constants (Figure 6, squares), consistent with the effect observed for WALP23 (Figure 3). In contrast, flop rates of C_6NBD-PG increased with increasing PG/PC ratios. Thus, it seems that, in TLE, the moderately inhibitory effect PE has on KALP23-induced flop of C_6NBD-PG is compensated for by a stimulatory effect of PG.

Sterols are main constituents of many membranes and are enriched in the plasma membranes of eukaryotic cells, which in contrast to the cytoplasmic membrane of bacteria do not

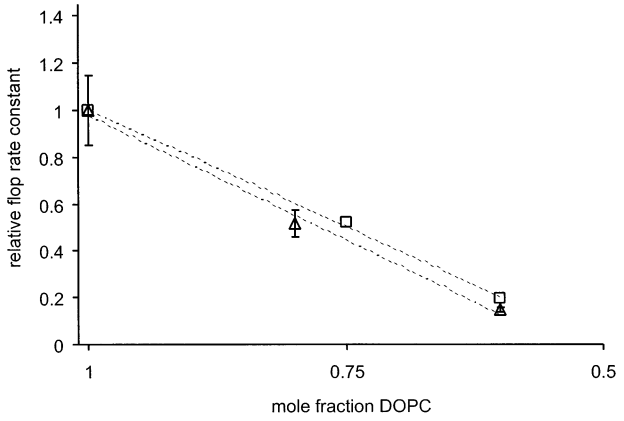

FIGURE 7: Relative K_{flop} values of peptide-induced flop of C_6NBD-PG vs the cholesterol content of the vesicles. Flop rate constants were determined in vesicles containing WALP23 (squares) or KALP23 (triangles) at a P/L molar ratio of 1:1000 and DOPC/cholesterol in various molar ratios as indicated on the *x*-axis and normalized to the flop rate constant in 100% DOPC. Averaged data points (triangles, $n = 3 \pm$ SD, and squares, $n = 2$) are connected by dashed lines for clarity.

show fast flip-flop of membrane phospholipids. Therefore, we tested the influence of cholesterol on peptide-induced flop of C_6NBD-PG in a DOPC matrix. With increasing cholesterol mole fractions (Figure 7), a strong, up to 6-fold, decrease in the rate of peptide-induced flop was observed for both KALP23 and WALP23, showing that apart from being modulated by the phospholipid composition of the membrane, transmembrane peptide-induced flop is also severely affected by the cholesterol content of the membrane.

DISCUSSION

Previously (*13*), we provided evidence for the hypothesis that, in the *E. coli* inner membrane, phospholipid translocation may be facilitated by the ubiquitous presence of α-helical transmembrane stretches of membrane proteins. In the present study, we present evidence that this phenomenon is not restricted to membranes with the phospholipid composition of *E. coli*. It is demonstrated that the rate of transmembrane helix-induced flop is modulated by the lipid composition of the membrane and strongly dependent on the nature of the translocating phospholipid analogue.

Examining the influence of the lipid composition of the bilayer, we showed that the rate of C_6NBD-PG flop, induced by both KALP23 and WALP23, was reduced with increasing amounts of DOPE. In a bilayer structure, this nonbilayer lipid causes an increase in lateral pressure in the hydrocarbon region (*19* and references cited therein) and an increased acyl chain order (*20*). Probably for the same reason, increasing amounts of cholesterol strongly inhibited flop. The main translocation barrier for phospholipid headgroups is the hydrocarbon core of the membrane. We conclude that a higher packing density in this region of the membrane results in decreased flop rates. Thus, the effects of DOPE and cholesterol presumably reflect a general effect on the physical properties of the bilayer.

The presence of increasing amounts of DOPG in DOPC vesicles enhanced the rate of KALP23-induced flop, whereas WALP23-induced flop was hardly affected. Although the molecular details causing this effect are as yet unclear, we think we can exclude the possibility of local enrichment of negatively charged phospholipids around the positively charged peptide, because C_6NBD-PG flop rates in DOPC vesicles are similar for WALP23 and KALP23.

Using different C_6NBD-labeled phospholipid analogues, striking differences between peptide-induced flop rates were observed. The negatively charged PG and PA analogues were translocated quickly, PE was translocated at a lower rate, and flop of PC and PS could not be observed under the conditions tested. This order of flop efficiency correlates with the total number of charges found on the probes. In PG and PA (anions) the phosphate group carries the negative charge. PE and PC have two charges (zwitterions), and PS has three (zwitterionic plus an extra negative charge). If the efficiency of flop of phospholipids depends on the "activation energy" of bringing the phospholipid headgroup in the hydrophobic interior of the bilayer, this energy is arguably higher when there are more charges on the headgroup. Possibly, negative charges are less inhibitory than positive charges, because the latter have to travel against the membrane dipole potential, which is positive inside the hydrophobic interior of the membrane (*21*). This potential arises from the orientation of dipoles at the lipid−water interface, on both sides of the membrane. The oriented water of hydration is thought to contribute most to this potential (*22*). Its value is thought to be in the range of +220 to +280 mV (*21*), which may explain the efficient flop of phospholipids with only negative charge, as compared to other phospholipid classes. The difference between PE and PC might be due the fact that the latter has a bigger headgroup (*23*).

Alternatively, one could argue that the phospholipids translocate as temporarily uncharged molecules, as suggested for translocation of anionic lipids driven by a transmembrane pH gradient (*24*). A strong pH dependency of peptide-mediated translocation of C_6NBD-PG, with higher flop rates at lower pH, should be expected if the flop rates are influenced by protonation. In contrast, the KALP23-induced translocation rate of C_6NBD-PG is ∼20% reduced at pH 5.5, as compared to pH 8.2 (unpublished observations), arguing against the possibility of translocation of phospholipids in the uncharged state.

The rate of WALP23-induced flop of C_6NBD-PG, both in TLE and DOPC, depended linearly on the peptide/lipid molar ratio. This was also observed for a lysine-flanked, somewhat longer peptide KALP31 [Ac-GKKL$(LA)_{12}$KKA-amide] in TLE (*13*). This linear relation indicates that the phospholipids are probably translocated through peptide monomers of both WALP and KALP, in membranes composed of both TLE and DOPC. The predominance of peptide monomers is in agreement with other observations (*25*).

The mechanism by which phospholipids cross a peptide-containing phospholipid double layer involves a lowering of the activation energy of phospholipid flop by the presence of peptide. It was shown (*15*, *26*) that the model peptides can influence the overall properties of the lipid bilayer, when present at a high peptide/lipid molar ratio (typically 1:50 or higher). At a typical peptide/phospholipid molar ratio of 1:1000, however, the *overall* physicochemical membrane properties are likely to be unaffected. We therefore propose that peptide-induced flop occurs via *local* defects near the peptide, the rate depending on the charge and volume of the phospholipid headgroup as discussed above. As a conse-

quence of the continuous thermal motion of the lipids and the discontinuity in lipid−lipid interactions on the peptide−lipid interface, a phospholipid may occasionally "slide along" the interface into the hydrophobic interior of the membrane and from there "surface" on the other side (*27*). Changing the lipid matrix can result in increased packing of the acyl chains through which the polar headgroup has to travel. This may increase the activation energy of flop and thus inhibit it.

In *E. coli*, phospholipid synthesis must be regulated in order to obtain the characteristic wild-type phospholipid composition. Indeed, the activity of, e.g., phosphatidylserine synthase (pss), one of the key enzymes involved in PE biosynthesis (*28*), has been demonstrated to be upregulated by the presence of anionic lipids (*29*) and particularly PG, the most abundant anionic phospholipid in *E. coli* (*30*). Interestingly, in the present study we observed that flop of C_6NBD-PG was inhibited by increasing PE concentrations and could be stimulated by increasing PG molar ratios. We speculate that this functions as an autoregulatory mechanism to keep the amount of PG in the inner leaflet of the membrane within certain limits, by downregulating translocation of PG when it is not abundant and upregulating flop when its concentration is high.

The finding that peptide-induced flop occurs in vesicles with a variety of lipid compositions suggests that the mere presence of transmembrane helices may account for the fast translocation of phospholipids in several other biomembranes, e.g., the ER membrane (*7*, *8*), other bacterial inner membranes (*5*, *6*, *18*), and the inner (*31*) and outer (*32*, *33*) membrane of the mitochondrion. However, it seems unlikely that all phospholipid classes commonly found in membranes are translocated via the transmembrane helical stretches of membrane proteins. In most studies, a lack of glycerophospholipid selectivity of flop in biogenic membranes (see, e.g., ref *4*) is observed, whereas peptide-induced flop as shown here is strongly dependent on the phospholipid headgroup. This suggests that apart from transmembrane helix-induced flop additional pathways are involved in phospholipid translocation, possibly via the putative flippase(s), which mediate energy-independent, fast phospholipid translocation.

The plasma membrane of eukaryotic cells displays only slow flip-flop although it contains a variety of transmembrane α-helical proteins. This slow flip-flop is consistent with the well-established phospholipid asymmetry between the two lipid monolayers (*34*). Loss of asymmetry, or more precisely exposure of aminophospholipids at the extracellular leaflet, is associated with several signaling events, including recognition by macrophages (*35*) and apoptosis (*36*). It is of interest to note that neither PS nor PC, which are both common phospholipids in eukaryotes (see, e.g., ref *37*), displayed helix-induced flop in our model system. This is consistent with their reported asymmetry across the eukaryotic plasma membrane.

In contrast to phospholipid translocation in the plasma membrane, flip-flop in the ER seems to be less tightly regulated. Interestingly, there is a gradual increase in the cholesterol content along the exocytotic pathway, the concentration being highest in the plasma membrane. In our experiments, cholesterol showed a strong inhibitory effect on helix-mediated flop, and therefore this increasing cholesterol content may provide a regulatory device to switch from constitutive flip-flop in the biogenic ER membrane to regulated phospholipid translocation in the plasma membrane.

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