Ultrafast Glycerophospholipid-selective Transbilayer Motion Mediated by a Protein in the Endoplasmic Reticulum Membrane*

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A relatively rapid transbilayer motion of phospholipids in the microsomal membrane seems to be required due to their asymmetric synthesis in the cytoplasmic leaflet. Marked discrepancies exist with regard to the rate and specificity of this flip-flop process. To reinvestigate this problem, we have used both spin-labeled and radioactively labeled long chain phospholipids with a new fast translocation assay. Identical results were obtained with both types of probes. Transbilayer motion of glycerophospholipids was found to be much more rapid than previously reported (half-time less than 25 s) and to occur identically for phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine. Such transport is nonvectorial and leads to a symmetric transbilayer distribution of phospholipids. In contrast, transverse diffusion of sphingomyelin was 1 order of magnitude slower. Phospholipid flip-flop appears to occur by a protein-mediated transport process displaying saturable and competitive behavior. Proteolysis, chemical modification, and competition experiments suggest that this transport process may be related to that previously described in the endoplasmic reticulum for short-chain phosphatidylcholine (Bishop, W.R., and Bell, R.M. 1985 Cell 42, 51–60). The relationship between phospholipid flip-flop and nonbilayer structures occurring in the endoplasmic reticulum was also investigated by 31P-NMR. Several conditions were found under which the 31P isotropic NMR signal previously attributed to nonbilayer structures is decreased or abolished, whereas transbilayer diffusion is unaffected, suggesting that the flip-flop process is independent of such structures. It is concluded that flip-flop in the endoplasmic reticulum is mediated by a bidirectional protein transporter with a high efficiency for glycerophospholipids and a low efficiency for sphingomyelin. In vivo, the activity of this transporter would be able to redistribute all changes in phospholipid composition due to biosynthetic processes between the two leaflets of the endoplasmic reticulum membranes within a time scale of seconds.

The endoplasmic reticulum of eukaryotic cells is the site of synthesis of several phospholipids including phosphatidylcholine, phosphatidylethanolamine, and in part phosphatidyserine. These synthetic activities are mostly located on the cytoplasmic leaflet of the membrane (1–3). Therefore translocation of newly synthesized phospholipids is likely to be necessary for proper biogenesis of the membrane. This suggest that a rapid transbilayer motion of phospholipids occurs in the endoplasmic reticulum (4).

Many studies have been devoted to the measurement of this flip-flop activity in the E.R. (1). Although all agree for a relatively rapid translocation of phospholipid in isolated microsomes as compared with many other membranes, discrepancies exist with regard to both the rate and the lipid specificity of the transverse diffusion process. Half-times ranging from 2–3 (5) to 45 min (6) have been measured using various methods. A much more rapid translocation of glycerophospholipids compared with sphingomyelin was found in one study (6) and not in another (7).

The mechanism of this rapid flip-flop is also a subject of controversy. Two proposals have been made in this regard. Bishop and Bell (8) found that the translocation of short chain PC is protein-mediated and suggested the occurrence of a PC transporter in the endoplasmic reticulum membrane. On the other hand Van Duijn et al. (9) have suggested that the nonbilayer structures present in microsomal membrane as detected by 31P-NMR (10) is responsible for the transverse diffusion.

In the present study, we have reinvestigated the rate, selectivity, and mechanism of the translocation of phospholipids in rough and smooth endoplasmic reticulum membranes. For this purpose we have used both spin-labeled and radioactive long chain phospholipids with various head groups in conjunction with a new lipid transport assay adapted to the measurement of rapid flip-flop processes. We have also studied in parallel the involvement of nonbilayer lipid structure in the microsomal membrane by 31P-NMR.

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin, diisopropylfluorophosphate, sodium dodecylsulfate, N-ethylmaleimide, trypsin, soybean trypsin inhibitor, glucose-6-phosphate, and mannose-6-phosphate were purchased from Sigma. Spin-labeled phospholipids 1-palmitoyl-2-(4-doxylpentanoyl)-sn-glycero-3-phosphocholine (SL-PC), phosphoserine (SL-PS), phosphoethanolamine (SL-PE), and (N-[4-doxylpentanoyl]-sphingosine)-1-phosphocholine (SL-PM) and the lyso derivative 1-[16-doxyl-stearoyl]-sn-glycero-3-phosphocholine (SL-LPC) were synthesized as described (11–13). Radioactive phospholipids were synthesized from sodium (1-14C)-butyrate purchased from ICN. The procedures to obtain 1-stearoyl-palmitoyl-2-[1-14C]-butanoyl-sn-glycero-3-phosphocholine (RL-PC) and N-[1-14C]-butanoyl)-sphingosine-1-phosphocholine (RL-

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Liver microsomes were prepared from male Wistar rats of 300–350-g body weight, which were starved for 16 h prior to slaughtering but had full access to water. Rough and smooth microsomes were isolated from rat liver as described (16). Membranes were resuspended at 1 mM MgSO₄, 50 mM Tris-HCl, 250 mM sucrose, pH 7.3, and pretreated with 4 mM diisopropylfluorophosphate before use in order to prevent phospholipase A₂ activity. Protein concentration was determined by the Coomassie Blue protein reagent (Pierce). The purity of rough and smooth microsome fraction was assessed by the specific activity of glucose-6-phosphatase (16) within inorganic phosphate determination according to Rouser et al. (17) and found to be similar to those of other preparations. The integrity of microsomal membranes was checked through the mannose-6-phosphatase activity in the presence and the absence of taurocholate (5, 18). A latency of 91% was found in agreement with previous results (5, 6, 18).

Measurement of Phospholipid Analog Translocation—In order to measure phospholipid flip-flop with a time resolution of 30 s, the following procedure was used. Translocation was initiated by adding membranes (final protein concentration, 0.8 mg/ml) to a concentrated suspension of radioactive or spin-labeled phospholipids in buffer. After various incubation times at 20 °C, 50-μl aliquots were taken and mixed by repetitive pipetting for 20 s with 50 μl of 10% SDS, bath-sonicated at 37 °C for 30 min, and centrifuged. Control experiments indicated that 90% of the filter material was recovered. Both BSA (outer leaflet analog) and SDS (inner leaflet analog) fractions were then assayed either by scintillation counting or by ESR using a Bruker ER-200d-SRC spectrometer. The total amount of probe was obtained from two control aliquots: one in which the membranes were incubated for 10 min with BSA before filtration allowing for total probe extraction and another in which the membranes were mixed with buffer in place of BSA allowing for total probe recovery in the SDS fraction. All data were corrected to take into account filter dead volume effects. To assess the extent of back translocation during the assay, we also measured SL-PC flip-flop in ER at 10 °C. A half-time of 3 min was found corresponding to an extent of translocation of 5% in 30 s. Because translocation occurs identically in both directions (see “Results”), this indicates that the extent of back translocation during the 30-s BSA extraction at 10 °C is of the order of 5%.

NMR—³¹P-NMR spectra were recorded at 20 °C in 10-mm sample tubes on a Bruker AMX 400 spectrometer operating at 160 MHz with WALTZ-16 proton decoupling during acquisition and a 2-s relaxation delay. 2-ml membrane samples at 35 mg/ml were used. NMR samples contained 20% D₂O (v/v) for deuterium locking. For samples containing dibucaine, the following procedure was used in order to ensure an
effector concentration similar to that used in phospholipid translocation experiments: membranes at 0.8 mg/ml protein concentration were supplemented with dibucaine at a concentration identical to that used in translocation assays, centrifuged, and resuspended at 30 mg/ml in part of the supernatant.

RESULTS

Transbilayer Motion of Spin-labeled Phospholipids in the Rough Endoplasmic Reticulum

In order to assess transbilayer diffusion in the rough endoplasmic reticulum membrane, we first incorporated spin-labeled phospholipids in the outer leaflet that subsequently underwent flip-flop to the inner leaflet. The results are shown in Fig. 1. A new translocation assay combining BSA extraction and rapid filtration allowed us to measure the probe phospholipid both in the outer leaflet (upper curves) and in the inner leaflet (lower curves) with a time resolution of ~30 s. For all glycerophospholipids a very rapid translocation is observed. For both SL-PC and SL-PS the probes initially incorporated in the outer leaflet are translocated to the inner leaflet with a half-time of 25 s (note that this should be considered as a maximum value due to the time resolution of our method) (Fig. 1, a and b), SL-PE is translocated slightly faster, having a half-time of 20 s (Fig. 1c). Such transbilayer diffusion appears to lead to a final transbilayer distribution of the phospholipids that is nearly symmetric. In contrast the translocation of SL-SM (Fig. 1d) occurs at an initial rate that is 1 order of magnitude slower than that found for glycerophospholipids (half-time, 4 min). This slow transbilayer diffusion leads in 30 min to a final transbilayer distribution that remains asymmetric with 75% of the SL-SM remaining in the outer leaflet (data not shown). Another phospholipid that displays a slow translocation is SL-LPC (Fig. 1e) because it undergoes only 5–10% translocation in 10 min.

Fig. 2 shows the dependence of the initial rate of phospholipid translocation upon concentration. All three glycerophospholipids show a similar saturable behavior with an apparent $K_m$ corresponding to a spin label mole fraction of 0.03 with regard to total phospholipids. The catalytic constants that can be calculated range from 37.5 nmol·min$^{-1}$·mg$^{-1}$ for SL-PC and SL-PS to 45 nmol·min$^{-1}$·mg$^{-1}$ for SL-PE. On the other hand, SM shows no evidence for such saturation. However, saturation would be difficult to detect due to the low rates of SM translocation.

To investigate whether the transbilayer diffusion process is vectorial, we also measured transbilayer diffusion of phospholipids from the inner leaflet to the outer leaflet of the rough endoplasmic reticulum membrane. Microsomal membranes were labeled with SL-PE and incubated in order to reach a stationary transbilayer distribution. BSA, which first extracted external phospholipids, was then added. The BSA incubation was continued for the indicated times in order to extract phospholipid back translocated to the outer leaflet. The results are shown in Fig. 3 for SL-PE and indicate that transbilayer diffusion occurs with similar characteristics in both directions. At

![Fig. 2. SL phospholipid dependence of transbilayer motion.](http://www.jbc.org/)

![Fig. 3. Inside-outside transbilayer motion of SL phospholipids.](http://www.jbc.org/)

![Fig. 4. Outside-inside transbilayer motion of RL phospholipids in rough ER.](http://www.jbc.org/)
Rapid Phospholipid Flip-Flop in Microsomes

Table I

| Treatment | Percentage of SL-PC transverse diffusion activity remaining | Mannose-6-phosphatase latency |
|-----------|-------------------------------------------------------------|-----------------------------|
| None      | 100                                                         | 91                          |
| Trypsin   | 68 ± 5                                                      | 92                          |
| NEM (20 mW) | 84 ± 10                                                   | 87.2                        |
| diC4PC (5 mW) | 100                                                      | 90                          |
| diC4PC (60 mW) | 71 ± 4                                                   | 91                          |

As shown in Fig. 4, the transbilayer diffusion of the radioactively labeled phospholipid, RL-PC and RL-SM, is identical to that of the corresponding SL-analogs. As an example at a 0.02 mole fraction, the rate of translocation of RL-PC and SL-PC were 22.1 ± 0.9 and 19.4 ± 1.0 nmol-min⁻¹-mg⁻¹, respectively. As shown in Fig. 5, the results of competition experiments in which translocation of RL phospholipids was studied in the presence of SL phospholipids. In the presence of increasing concentrations of SL-PE, the initial rate of transbilayer diffusion of RL-PC was progressively slowed down indicating competition for transport (Fig. 5a). A smaller but definite effect was found for RL-PC in the presence of SL-SM, suggesting that competition also occurs between these two phospholipids (Fig. 5b). In contrast, no effect of SL-LPC on the flip-flop of RL-PC was found, demonstrating an absence of competition between these two phospholipids (Fig. 5c). This also indicates that the effects shown in Fig. 5 (a and b) are indeed due to competition and not to a membrane fluidity variation associated with the spin label added in excess.

Relationship of Long Chain Phospholipids Flip-Flop and of the Short Chain PC Transporter—Bishop and Bell (8) have demonstrated a relatively rapid protein-mediated transport of diC4PC in endoplasmic reticulum membranes. Both trypsin and N-ethylmaleimide treatment of ER membranes have been demonstrated to decrease diC4PC transport. As shown in Table I, both treatments also diminished the transbilayer diffusion of SL-PC in ER membranes at a lower extent than that found for diC4PC transport (8). Competition for transport between SL-PC and diC4PC was also measured. The presence of an excess of diC4PC leads to a small reduction of the flip-flop of SL-PC in rough ER.

Relationship between SL Phospholipid Flip-Flop and Nonbilayer Structure in the ER Membrane—In order to investigate the possible relation between phospholipid flip-flop and nonbilayer structures in the ER, we have carried out parallel transbilayer diffusion measurements of SL phospholipids and 31P-NMR experiments (Fig. 6) under multiple conditions. Fig. 6a shows the 31P-NMR spectrum of rough endoplasmic reticulum membranes at 20 °C. It is comparable with that obtained by other authors (19). Above the powder spectrum characteristic of the bilayer organization of the membrane, a number of more or less narrow peaks are observable. The three narrowest peaks (line widths 20–25 Hz) are attributable to inorganic phosphate and monoester phosphate metabolites (19, 20). These are resistant to extensive washing of the membrane and are therefore located in the lumen of microsomes. The broader isotropic peak (150–200 Hz) has been previously attributed to translocation as well as to perform competition experiments using phospholipids with different head groups.

Comparison and Competition between SL Phospholipids and RL Phospholipids—Besides SL phospholipids, we have also used radioactive phospholipids carrying a C4 β-chain to study transbilayer motion in endoplasmic reticulum membranes. This allowed us to assess the effect of probe structure upon
to nonbilayer structures present in the ER membranes (9, 21).

Two distinct treatments of the microsomes were found to lead to a decrease of the nonbilayer peak. This included addition of dibucaine as already reported by De Kruijff et al. (21) and washing of the membrane with 3% (v/v) Me2SO. As shown in Fig. 6 (b and c), the isotropic 100 Hz wide peak almost completely disappeared in both treated membranes. Me2SO treatment also led to the disappearance of the narrow metabolic peaks, presumably due to transient permeabilization of the vesicles. Despite of these NMR changes, we found that neither dibucaine nor Me2SO treatment affected to any extent the transbilayer diffusion of SL phospholipids as shown in Fig. 6 (f and g) for SM and PE. Control enzymatic measurements indicated that the integrity and the polarity of the vesicles was preserved at 90% after these treatments.

We have also compared rough and smooth microsomes with regard to both 31P-NMR and phospholipid flip-flop. The 31P-NMR spectrum of smooth microsomes is characterized by a much decreased nonbilayer peak as compared with rough microsomes. In contrast, it was found that transbilayer diffusion of SL phospholipids was identical in both microsomal fractions (compare Fig. 6, e and h).

**DISCUSSION**

The passive transverse diffusion of phospholipids in lipid bilayers or in most biological membrane usually occurs within
time scales of hours or days (for review see Zachowski (22)). All studies devoted to the measurement of flip-flop in ER membranes have indicated a more rapid process. However, half-times ranging from 3 to 45 min have been reported for PC. In addition, the transverse diffusion of SM has been found to be similar to that of PC in one study (7) and much slower in another (6). Such discrepancies may in principle arise from two origins. The first is related to the type of phospholipid probe used for the assay of transbilayer diffusion. Although a few studies have used natural phospholipids (5, 6), others have used either spin-labeled phospholipids carrying one short chain (7) or soluble phospholipids with two short chains (8, 23). Although such probes probably behave qualitatively as natural phospholipids, quantitative differences in their transverse diffusion rates cannot be excluded. A second origin for the differences may come from the slow time scales of the methods used to assay phospholipid transport. Most approaches have measured the disappearance of external leaflet phospholipid using either BSA extraction or phospholipid exchange protein extraction followed by a slow separation step using centrifugation. An exception is the study by Bishop and Bell (8), which used a rapid filtration assay to recover external soluble short chain phospholipids. However, it is also possible that such short chain phospholipids have slower transport rates than long chain phospholipids because their diffusion from the aqueous phase to the membrane may be a limiting factor.

In the present study, we have attempted to take these two potential limitations into account. First, we have used two types of phospholipid probe carrying a long chain fatty acid at the sn-1 position and a spin-labeled (7) or radioactively fatty acid at the sn-2 position. The fact that identical results are obtained with both probes gives us confidence that these are relatively faithful reporters of natural phospholipids. Second, in order to assay the translocation of these membrane bound phospholipids with a rapid time scale, we have adapted the standard BSA extraction procedure into a rapid filtration assay having a time resolution of ~30 s.

With these improvements, it is shown that for glycerophospholipids, the transbilayer diffusion is even more rapid than previously concluded. Indeed, half-times for diffusion of the order of 25 s were found for PC, PE, and PS. Considering that this value corresponds to the time resolution of our method, it is possible that this flip-flop might be even faster. This rate is about 10 times more rapid than the fastest transbilayer diffusion rates previously reported in the ER (8). Such rapid flip-flop can occur in both directions with a similar efficiency.

Using identical spin-labeled phospholipids, Herrmann et al. (7) found much lower transverse diffusion rates of glycerophospholipids in ER, with half-times of 20 min at 37 °C. These authors used a slow BSA extraction assay (1-min incubation followed by 2.5-min centrifugation) and measured only the disappearance of external leaflet phospholipids. To understand this discrepancy, we have performed control experiments using our method but with the 3-min incubation time of the labeled microsomes with BSA (data not shown). Under such conditions, an apparent half-time of 23 min for SL-PC flip-flop was found at 37 °C in agreement with Herrmann et al. (7). This suggests that the apparently slow transverse diffusion reported by these authors was due to the use of a translocation assay with too long a time scale compared with the 25-30-s half-time of glycerophospholipid transverse diffusion in ER.

There appears to be little specificity in the very fast transverse diffusion of glycerophospholipids, with PE transport being slightly more rapid than PC and PS. This result is in contrast with the very high selectivity of the transbilayer diffusion of SM and LPC. SM diffuses transversally an order of magnitude slower than glycerophospholipids. The relative differences in rates found for PC and SM are in agreement with those found by Zilversmit et al. (6). The other phospholipid that displays a slow flip-flop in the ER is the spin-labeled analog of LPC. LPC is an important intermediate in the phospholipid biosynthetic routes of the ER. Our results contrast with those of Kawashima and Bell (23), who found a very rapid transport of a short chain LPC in microsomes. Of course, it cannot be excluded that the presence of the nitroxide group perturbs LPC transport, although this does not appear to be the case for glycerophospholipids.

This rapid flip-flop of glycerophospholipids has consequences in relation to the topography of phospholipids synthesis in ER. Bishop and Bell (8) pointed out that the rapid PC transport that they observed (half-time, 5 min) was sufficient to ensure an even biogenesis of both membrane leaflets in the ER, because it was more efficient than the main biosynthetic routes for phospholipids that occur on the cytoplasmic surface (24). Our finding of an even faster flip-flop of all glycerophospholipids further suggests that transbilayer diffusion can in fact redistribute within seconds any change in composition due to the activity of biosynthetic enzymes. In agreement with this, several authors reported that appearance of phospholipids on one leaflet occurred very rapidly after their synthesis on the other leaflet (4).

The fast transverse diffusion of glycerophospholipids appears to promote a nearly symmetric transmembrane distribution of all head group species between the two leaflets. As pointed out by Herrmann et al. (7), the average size of microsomal vesicles corresponds to a slight area difference between the two leaflets so that a symmetric distribution corresponds to ~45% of the phospholipid on the internal leaflet. Our results indicate that PC, PS, and PE are distributed respectively to 40, 40, and 45% in the inner leaflet. These values may be slightly underestimated due to the occurrence of a very limited back translocation during the translocation assay (see “Experimental Procedures”) as well as to a possible leakiness of some of the membranes (in view of the 91% mannose-6-phosphatase latency). Therefore our results indicate that the glycerophospholipid transverse distribution is symmetric or nearly symmetric in the ER. Studies that assayed the endogenous phospholipid asymmetry in the ER found a more asymmetric distribution of glycerophospholipids both in rough and smooth ER (25–27). The discrepancy may be explained by the use of methods with a slow time scale in these reports as compared with the very rapid flip-flop rates in the ER.

Our data also allow us to have an insight into the molecular mechanism of the rapid phospholipid flip-flop in the ER. The results described above indicate that this mechanism displays a strong structural selectivity for the glycerol backbone and for the β-position but exhibits little discrimination between different head groups. Furthermore, our data show that this transbilayer motion is not a simple diffusion but a transport process involving discrete sites. Indeed for all three glycerophospholipids, a saturable behavior was observed as a function of phospholipid concentration. Competition for transport could be detected between different glycerophospholipids. Competition is also observed between PC and SM, suggesting that the latter is also transported by the same pathway, although with a much lower efficiency. Indeed it must be emphasized that the diffusion of SM in ER remains significantly faster than in other systems (12). On the other hand, no competition was found between glycerophospholipids and LPC. These data confirm that a protein-mediated transport of phospholipids occurs in the ER membrane. It appears to be an ATP-independent (data not shown) bidirectional transport. Such a transport activity
has already been demonstrated by Bishop and Bell (9) with short chain phospholipids and reconstituted in liposomes by Baker and Dawidowicz (28). Several of our results suggest that both transport systems are related without definitely proving their identity. Both appear to be sensitive to N-ethylmaleimide and to trypsin although to a different extent. There exists a definite inhibition of spin-labeled phospholipids transport in the presence of diC4PC, suggesting competitive behavior. The limited extent of this inhibition may be due to the fact that the membrane-bound state of the SL phospholipids promotes a much higher affinity for the transport sites than the soluble nature of diC4PC. Our data do not constitute a definitive proof for helpful discussions.

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