Facilitated Phospholipid Translocation across Vesicle Membranes Using Low-Molecular-Weight Synthetic Flippases

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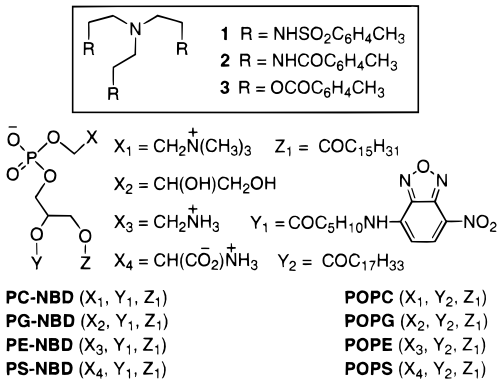
Received September 14, 1999

The distribution of phospholipids across most, if not all, cell membranes is asymmetric. For example, around 80% of the sphingomyelin and phosphatidylcholine in erythrocyte membranes is localized in the exo (outer) leaflet, whereas 80−85% of the phosphatidylethanolamine and >96% of the phosphatidylserine is in the endo (inner) leaflet.[1] A cell consumes significant amounts of chemical energy to maintain these asymmetric distributions, as they control a range of important processes such as enzyme activation, membrane fusion, coagulation, and apoptosis.[1,2] It is generally accepted that spontaneous phospholipid translocation (also known as flip-flop) across a bilayer membrane is a very slow process and is facilitated by membrane-bound enzymes known as translocases or flippases. In the case of erythrocyte membranes, a number of different phospholipid translocation activities have been identified, including an ATP-dependent inward translocase with selectivity for aminophospholipids, a less-selective ATP-dependent outward translocase, and a Ca^{2+}-dependent nonselective scramblase.[2] Other cell types contain additional classes of flippases.[3] Some of the flippase proteins have been cloned and shown to have in vitro activity, but presently it is not known how translocation works at the molecular level.[4] Mechanistic features that have been discussed include protein pores[5] and localized membrane defects;[6,7] however, it is not clear if these models apply to flippases that can discriminate between phospholipid headgroups.

At present, the literature contains few examples of chemically induced phospholipid translocation.[1,7,8] Rationally designed, synthetic flippases are likely to be useful mechanistic models of their more complex biological counterparts. They also may find employment as pharmaceuticals or as chemical tools for biological membrane research. The only previous example of an artificial flippase was reported by Moss, Ringsdorf, and co-workers,[9] who showed that a high-molecular-weight, hydrophobically modified polymer can facilitate phospholipid translocation. The data are in favor of a membrane disruption mechanism, which suggests that this system is unlikely to discriminate between phospholipid headgroup structures. Here, we report that the low-molecular-weight tris(aminoethyl)amine derivatives **1** and **2** (Scheme 1), which act as receptors for phosphate anions in organic solvents,[10] are also able to selectively facilitate the translocation of fluorescent phospholipid analogues across the membranes of surface-differentiated vesicles.

Scheme 1



Membrane translocation was measured using the 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)/dithionite quenching assay.[11,12] To briefly summarize, dithionite ($S_2O_4^{2-}$) selectively reduces the nitro groups on the fluorescent NBD-containing phospholipids (NBD-lipids) shown in Scheme 1 and produces nonfluorescent amine products. Since dithionite diffuses very slowly through vesicle membranes, it will react and quench the fluorescence of an NBD-lipid only in the membrane outer leaflet. Initial studies were conducted at pH 7.4 and 25 °C using vesicles made from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (Scheme 1). The inward translocation experiment is initiated by adding to the POPC vesicles a small aliquot of PC-NBD (0.5 mol % of total phospholipid) in ethanol, which readily inserts into the outer leaflet of the vesicle membrane. Subsequent addition of quenching dithionite allows the amount of exo PC-NBD to be determined. Lysis of the vesicles using detergent allows the dithionite to gain access to the remaining PC-NBD, and thus the percent exo PC-NBD can be computed. The inward translocation experiment starts with 100% endo PC-NBD and progresses to an equilibrium value of around 65%.[11,12] As shown in Figure 1, the background rate for inward translocation of PC-NBD in POPC vesicles is very slow, with a translocation half-life of much greater than 3 h. Addition of amide **2** or control ester **3** produces a very minor enhancement of inward translocation, whereas the presence of sulfonamide **1** produces more than a 100-fold increase. Outward translocation experiments show the same trend (Figure 1).[13] Concentration studies show that the rate of translocation increases as the concentration of **1** or **2** is raised. Also, the same translocation rates were observed with 120- or 200-nm vesicles. Furthermore, control experiments indicate that adding compounds **1** or **2** (38 μM) to POPC vesicles (25 μM) induces (1) no leakage of encapsulated calcein dye, (2) no change in vesicle size as judged by dynamic light scattering, and (3) no change in membrane fluidity as reflected by the excimer/monomer ratio produced by POPC vesicles that include 10% 1-palmitoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine.[14]

It appears that the sulfonamide **1** is able to form a transient hydrogen-bonded complex with the PC-NBD headgroup as shown

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10.1021/ja9933285 CCC: $18.00
Published on Web 12/03/1999

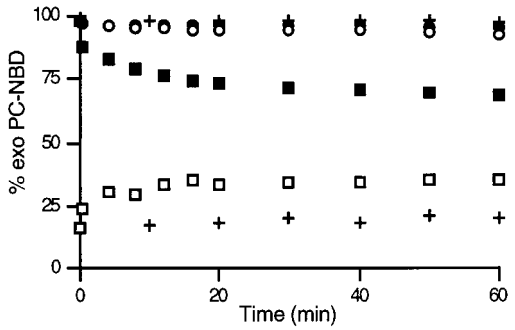

Figure 1. Percent of PC-NBD in the exo leaflet of POPC vesicles (25 μM phospholipid) at 25 °C and pH 7.4. Inward translocation induced by addition of 50 μM of **1** (■), **2** (○), and **3** (●) to exo-labeled vesicles. Outward translocation induced by addition of 38 μM of **1** (□) to predominantly endo labeled vesicles.[13] The background inward and outward translocation observed in the absence of added flippase is represented by crosses.

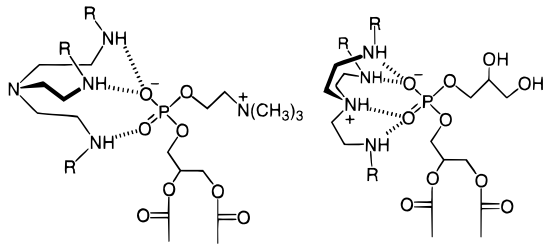

Figure 2. Postulated complexes between synthetic flippases **1** or **2** and the headgroups of PC-NBD and PG-NBD.

Table 1. Half-Lives for Inward Translocation of NBD-Lipids Induced by Synthetic Flippases

	NBD-lipid (translocation half-life /min)[b]	
vesicle composition[a] (25 μM total phospholipid)	flippase **1** (38 μM)	flippase **2** (38 μM)
POPC	PC-NBD (4)	PC-NBD (>180)
POPG	PG-NBD (6)	PG-NBD (2)
POPS	PS-NBD (30)	PS-NBD (>180)
POPC/POPG (1:1)	PC-NBD (14)	PC-NBD (>180)
	PG-NBD (4)	PG-NBD (2)
POPC/POPS (1:1)	PC-NBD (18)	PC-NBD (>180)
	PS-NBD (40)	PS-NBD (>180)
POPC/POPE (1:1)	PC-NBD (>120)	PC-NBD (>120)
	PE-NBD (>120)	PE-NBD (>120)
POPE/POPG (1:1)	PE-NBD (>120)	PE-NBD (120)
	PG-NBD (>120)	PG-NBD (60)
POPE/POPS (1:1)	PE-NBD (>240)	PE-NBD (>240)
	PS-NBD (240)	PS-NBD (240)

[a] Primarily unilamellar vesicles (mean diameter 120 nm) that initially contain NBD-lipid (0.5 mol %) only in the exo leaflet. [b] Time taken to reach halfway to equilibration. Estimated uncertainty, ± 33%. Background translocation half-life for all NBD-lipids is ≫180 min, except PG-NBD which is ~120 min. T = 25 °C, pH 7.4 buffer (5 mM TES, 100 mM NaCl).

in Figure 2. As a consequence, the polarity of the zwitterionic phosphocholine headgroup is reduced, which enhances translocation into the lipophilic interior of the bilayer. The NH residues in amides are about 5 pK_a units less acidic than those in sulfonamides,[15] and amide **2** is known to bind $H_2PO_4^-$ in acetonitrile about 5 times more weakly than sulfonamide **1**.[10] Thus, the small translocation enhancement observed for amide **2** is attributed to its weaker affinity for the PC-NBD headgroup.

To learn more about the selectivity of the translocation process, competition experiments were conducted using mixed vesicle systems. For example, the inward translocation half-lives for PC-NBD and PG-NBD were determined in vesicles composed of 1:1 POPC/1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (POPG). As summarized in Table 1, both **1** and **2** are selective translocators of PG-NBD over PC-NBD. The >90:1 selectivity of amide **2** is particularly striking. The high translocation rates observed with PG-NBD are rationalized in terms of the ion-pair complex shown in Figure 2, which indicates that charge neutralization is an important factor in this system. However, charge neutralization alone is not sufficient because the control ester **3** induces little or no translocation enhancement in any of the systems listed in Table 1. The longer translocation half-lives seen with PS-NBD are in agreement with the complexation models shown in Scheme 1. The PS headgroup is a triple ion with a net negative charge; it is significantly more polar than the PG headgroup and thus harder to extract into the lipophilic membrane interior. The data in Table 1 provide additional mechanistic insights. For example, the half-life for inward PC-NBD translocation induced by **1** in pure POPC vesicles is about 4 times faster than the PC-NBD translocation observed in mixed 1:1 POPC/POPG or POPC/1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (POPS) vesicles. The POPG and POPS appear to be inhibitors of PC-NBD translocation, presumably because the anionic PG or PS headgroups sequester the protonated form of **1** at the bilayer surface.

Even more dramatic is the inhibitory effect of zwitterionic 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanol (POPE). The half-life for inward PC-NBD translocation induced by **1** in mixed 1:1 POPC/POPE vesicles is more than 30-fold slower than that observed in pure POPC vesicles. Similarly, the half-life for PG-NBD translocation induced by **1** or **2** in mixed 1:1 POPE/POPG vesicles is about 20 times slower than PG-NBD translocation in pure POPG vesicles. Outward translocation experiments also show the same trends (data not shown). Further study is needed to clearly establish the reason for this inhibitory effect, but at present the most obvious explanation is that the ammonium residues in the POPE form intermolecular hydrogen bonds with the neighboring phospholipid headgroups[17] and inhibit formation of the kinetically active complexes shown in Figure 2.

In summary, compounds **1** and **2** are able to selectively facilitate the translocation of phospholipid analogues across the membranes of surface-differentiated vesicles in the order phosphatidylglycerol > phosphatidylcholine > phosphatidylserine > phosphatidylethanolamine. Therefore, we classify these compounds as low-molecular-weight synthetic flippases. More preorganized versions of **1** and **2** are known to have increased anion binding affinities,[16] which suggests that it should be possible to prepare more efficient second-generation flippases with interesting chemical and biological properties. The molecular recognition of phospholipid headgroups is an important but surprisingly neglected research topic in supramolecular chemistry that warrants greater attention.

Acknowledgment. This work was supported by the National Institutes of Health (GM 59078) and the University of Notre Dame (Molecular Biosciences Fellowship to J.M.B.).

Supporting Information Available: Experimental procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA9933285

(13) The outward translocation experiment starts with vesicles containing fluorescent PC-NBD predominantly in the endo leaflet. This is achieved by treating vesicles that have an even distribution of 0.5 mol % PC-NBD with dithionite, which reacts and quenches the PC-NBD in the exo leaflet. Excess dithionite is removed by filtration, which leaves vesicles that have no exo PC-NBD; however, by the time the filtration is complete and the translocation assay is started there is a moderate amount (<20%) of exo PC-NBD. The outward translocation experiment is susceptible to artifacts due to vesicle leakage and multilamellarity. In this study, the vesicles were a mixture of mainly unilamellar with some multilamellar (see Supporting Information), which is why the outward translocation experiment shown in Figure 1 does not reach the expected equilibration point for unilamellar vesicles of around 65% exo.[11,12] The observation that externally added flippase can promote outward translocation implies that the lipophilic flippase is able to rapidly diffuse across the vesicle bilayer and facilitate translocation in both directions.

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