Facilitated Phosphatidylcholine Flip-Flop Across Erythrocyte Membranes Using Low Molecular Weight Synthetic Translocases

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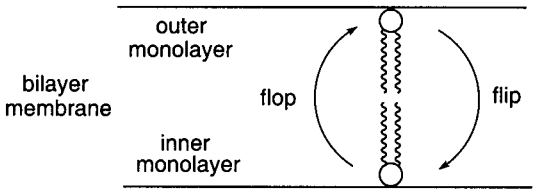
Abstract: The transmembrane distribution of phospholipids plays an important regulatory role in human erythrocytes. Membrane-bound translocase enzymes maintain an asymmetric phospholipid distribution across the membrane monolayers by promoting transmembrane diffusion or flip-flop. Mechanistic understanding of the flip-flop process is weak at the molecular level. Recently, we discovered that amide and sulfonamide derivatives of tris(aminoethyl)amine facilitate phospholipid flip-flop across vesicle membranes; that is, they act as low molecular weight, synthetic translocases. In this report, NMR evidence is provided that suggests that the synthetic translocases work by forming a hydrogen-bonded complex with the phosphocholine headgroup which decreases headgroup polarity and promotes diffusion across the lipophilic interior of the membrane. Also cell morphology and fluorescence probe methods are used to show that these synthetic translocases facilitate phosphatidylcholine flip-flop across erythrocyte membranes. Addition of a small amount of dilauroylphosphatidylcholine to erythrocytes produces echinocyte morphology which takes days to revert back to the original discocyte shape. The rate of return is significantly accelerated by the presence of the synthetic translocases. The synthetic translocases facilitate inward-translocation (flip) of the fluorescent phosphatidylcholine probe, 1-palmitoyl-2-(*N*-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]aminohexanoyl)-*sn*-glycero-3-phosphocholine (PC-NBD).

Introduction

In general, cell membranes have an uneven distribution of phospholipids at each membrane surface. For example, most of the aminophospholipids (phosphatidylserine and phosphatidylethanolamine) are found in the inside monolayer of animal plasma membranes, whereas most of the zwitterionic phospholipids (phosphatidylcholine and sphingomyelin) are localized in the outer monolayer.[1] The asymmetric phospholipid distributions control a number of biological functions, such as blood coagulation, cell clearance, membrane fusion, and apoptosis.[2] There is good evidence that the uneven distribution is maintained by membrane-bound enzymes, known generally as "translocases" or "flippases", which catalyze the inherently very slow translocation or "flip-flop" of phospholipids from one side of the membrane to the other (Scheme 1). There appear to be several types of translocase enzymes including a ubiquitous Mg^{2+}-ATP dependent aminophospholipid flippase that selectively catalyzes the inward transport of phosphatidylserine and phosphatidylethanolamine, an ATP dependent floppase that moves phospholipids outward with little headgroup specificity, and a Ca^{2+} dependent scramblase that destroys the bilayer asymmetry.[3,4] At present there is very little mechanistic understanding of enzymatic translocase action. For example, it is not known if the enzymes specifically interact with the polar lipid headgroups, or if they generate pore-like structures.[5]

Scheme 1. Phospholipid Translocation or Flip-Flop



We are attempting to develop synthetic, rationally designed translocases for use as pharmaceuticals or as chemical tools for biological membrane research.[6,7] We recently demonstrated that sulfonamide **1** (Scheme 2) can facilitate phosphatidylcholine translocation across vesicle membranes.[8] We proposed that this

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(4) In this paper we use the following terms: Translocase refers to the general family of phospholipid translocation catalysts; Flippase refers specifically to catalysts that promote inward translocation; Floppase refers to catalysts that promote outward translocation; and a Scramblase promotes translocation in either direction.

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(6) Enhanced phospholipid translocation has been achieved using high concentrations (>5 mM) of membrane-disturbing chemicals: Schwichtenhövel, C.; Deuticke, B.; Haest, C. W. M. *Biochim. Biophys. Acta* **1992**, *1111*, 35−44. Schneider, E.; Haest, C. W. M.; Plasa, G.; Deuticke, B. *Biochim. Biphys. Acta* **1986**, *855*, 325−236.

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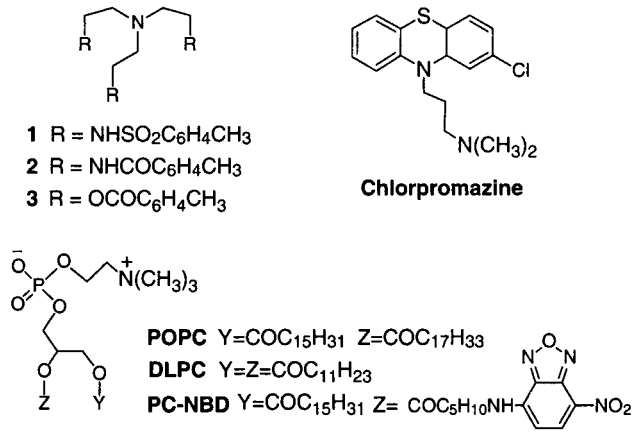
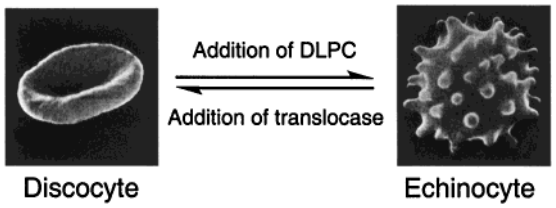
Scheme 2



Scheme 3. Discocyte/Echinocyte Equilibrium



low molecular weight synthetic translocase[4] works by forming a hydrogen-bonded complex with the phosphocholine headgroup which decreases headgroup polarity and promotes diffusion across the lipophilic interior of the membrane.[8] In this present contribution, we provide NMR data that support this supramolecular mechanism. More importantly, we demonstrate the first example of rationally designed synthetic translocase activity in cell membranes.[7] Visual evidence for enhanced transmembrane diffusion was gained from erythrocyte morphology studies. Human erythrocytes (red blood cells) are an ideal biological system for translocase studies because their morphology is very sensitive to phospholipid distribution.[9] The normal erythrocyte shape is a biconcave disk termed discocyte. As explained by the bilayer-couple hypothesis,[10] externally added phosphatidylcholine intercalates into the outer monolayer of a discocyte membrane, expands it, and produces outward-buckling of the monolayer. This forms an echinocyte (Scheme 3) which is a sphere covered with regularly spaced spicules, a shape that is maintained until the added phosphatidylcholine equilibrates across the membrane which takes days. We find that the rate of return from phosphatidylcholine-induced echinocyte morphology is significantly accelerated by the presence of synthetic translocases **1** and **2**, which promote phosphatidylcholine migration across the cell membrane.

Results

pK_a Determinations. Compounds **1**−**3** are only moderately soluble in pure water (solubilities ∼100 μM). Therefore, the pK_a* values of their protonated forms were determined by potentiometric methods in 9:1 methanol−water,[11] and found to be **1** (4.4), **2** (5.7), and **3** (4.2).[12] This suggests that compounds **1**−**3** exist predominantly as their free bases at the neutral pH used in this study.

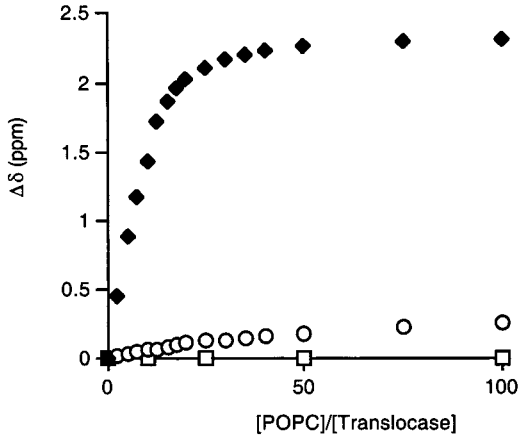


Figure 1. Change in synthetic translocase chemical shift ($\Delta\delta$) upon addition of POPC, in $CDCl_3$ and at 25 °C. Change in NH signal for **1** (filled diamonds), NH signal for amide **2** (open circles), and NCH_2 signal for ester **3** (open squares).

NMR Binding Studies. The ability of compounds **1**−**3** to complex with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) in $CDCl_3$ at 25 °C was evaluated using ^{1}H NMR spectroscopy. Titration isotherms were generated by adding aliquots of POPC to solutions of **1**, **2**, or **3** (Figure 1), and association constants were extracted by fitting the curves to a 1:1 binding model using iterative computer methods.[13] The 1:1 binding model is valid because dilution studies showed no evidence for significant self-aggregation of the POPC or compounds **1**−**3**. Also in the case of **1**/POPC the 1:1 binding stoichiometry was confirmed by a Job plot (not shown). The order of observed association constants is **1** (860 M^{-1}) > **2** (20 M^{-1}) > **3** (1 M^{-1}), which is in agreement with previous measurements of relative anion binding affinities in organic solvents.[14] A comparison of the ^{1}H NMR spectra of POPC alone, a 1:1 mixture of **1**/POPC, and **1** alone (Figure 2) reveals that the NH chemical shift for **1** and the headgroup signals for POPC change significantly when the two compounds form a complex. This is consistent with the supramolecular complex shown in Scheme 4.

Aggregation and Hemolysis Properties. The following critical micelle concentrations (CMCs) were determined using the fluorescent probe, rhodamine 6G chloride:[15] **1** (45 μM), **2** (45 μM), and **3** (75 μM). These CMC values are relatively low;[16] however, they are all higher than the 35 μM concentrations employed in these studies. Furthermore, hemolysis measurements showed that at 35 μM, compounds **1**−**3** induce only a few percent of hemoglobin leakage over 2 h.

Erythrocyte Morphology Studies. According to the bilayer-couple hypothesis,[10] echinocyte formation occurs when externally added amphiphile (as little as 0.7% of total phospholipid) intercalates in the outer monolayer of a discocyte membrane (Scheme 3). Conversely, if the added amphiphile accumulates in the inner monolayer, the subsequent inward-buckling of the monolayer leads to an inverted-mouth shape, termed stomatocyte.[10] These different cell morphologies can be identified using

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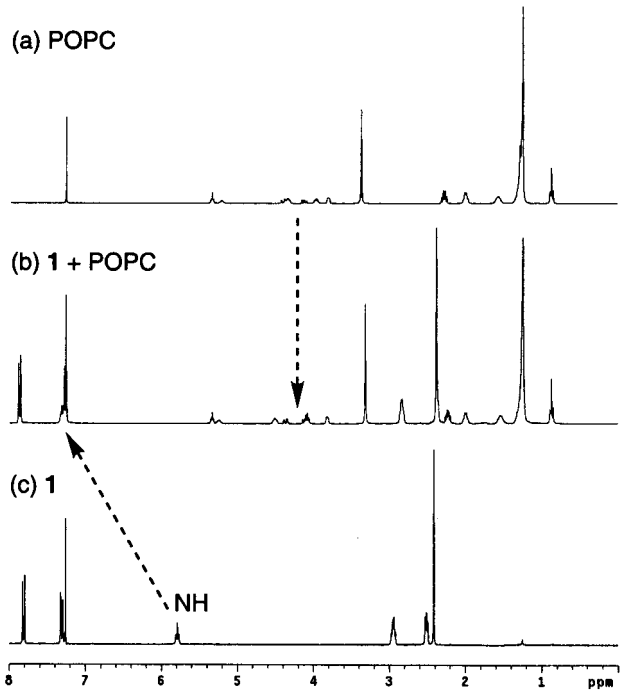
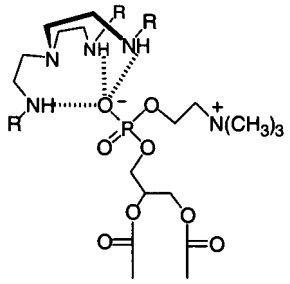


Figure 2. ^{1}H NMR spectra in $CDCl_3$.

Scheme 4



a light microscope. A quantitative evaluation is gained by using the morphological indexing scale described by Bessis.[9,17] Each cell in a large population is given a score based on its appearance. Discocytes are given a score of 0. Echinocytes may range from +1 to +5 depending on the number of spikes found on the surface. The number of invaginations determine the −1 to −4 score of stomatocytes. The morphology index is an average score for all the cells in a 100-cell population.

As shown by the morphological index curves in Figure 3, the presence of compounds **1**, **2**, or **3** (35 μM) at neutral pH induces erythrocytes to assume slightly stomatocyte shapes over a 5 h period. The order of effectiveness is **1** ∼ **2** > **3** although the differences are small. The same results are obtained if the experiment is run in the presence of vanadate, a known inhibitor of the endogenous aminophospholipid flippase (data not shown).[18,19] This suggests that compounds **1**−**3** induce stomatocytosis by slowly accumulating in the inner monolayer of the cell membrane. The slightly higher effectiveness of compounds **1** and **2** is attributed to their ability to also cotransport some phospholipid (most likely phosphatidylcholine and sphingomyelin) to the inner monolayer.

Compounds **1**−**3** were evaluated for their abilities to promote the reversion of echinocytes back to discocytes (Scheme 3).

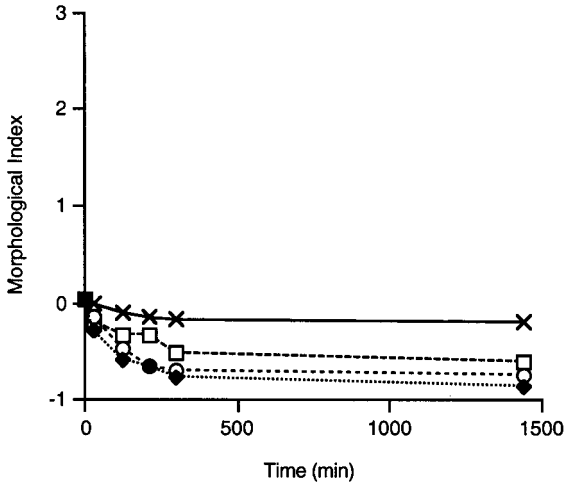


Figure 3. Change in morphological index for erythrocytes treated with 35 μM of sulfonamide **1** (filled diamonds), amide **2** (open circles), ester **3** (open squares), and no addition (crosses) at pH 7.4 and T = 37 °C. Each point is the average of six independent runs, with an uncertainty of ±0.15 morphological index units.

Echinocytes (Figure 4a) were formed by adding 35 μM of exogenous dilauroylphosphatidylcholine (DLPC) and then different batches of the echinocytes were treated with 35 μM of compounds **1**−**3**. After incubation for 2 h, a small fraction of echinocytes had returned to disk shape in the presence of ester **3** (Figure 4b), a larger proportion in the presence of amide **2** (Figure 4c), and essentially all of the cells had returned to discocyte shape in the presence of sulfonamide **1** (Figure 4d). Morphology index curves (Figure 5) also show that the order of echinocyte to discocyte reversion is **1** > **2** > **3**. Essentially identical curves were obtained with cells that had been treated with vanadate, an aminophospholipid flippase inhibitor.[18,19] A control experiment was conducted using chlorpromazine (Scheme 2), an amphiphilic amine that is well-known to rapidly induce stomatocytosis.[20] At 35 μM, chlorpromazine has little effect (Figure 5) although at higher concentrations it converts echinocytes into stomatocytes in less than 15 min. This latter observation agrees with literature reports that stomatocyte-forming amphiphiles can reverse the effects of echinocyte-forming amphiphiles.[21,22] The antagonism is due to the two different amphiphiles independently intercalating and expanding the opposing monolayers in the erythrocyte membrane.

Morphology index curves for reverse addition experiments are shown in Figure 6. In this case, the disococytes were treated with one of the translocase candidates (**1**, **2**, or **3**) followed 15 min later by DLPC, which rapidly induced echinocytosis. Under these conditions, the subsequent return to discocyte was in a slightly different order, that is, compounds **1** and **2** had the same effectiveness, which was much better than **3**. After 24 h, all of the above cell/synthetic translocase systems had equilibrated and reached about the same final morphology index value of −0.8 (i.e., slightly stomatocytic).

Inward Translocation of Fluorescent Phosphatidylcholine Probe. The synthetic translocases **1** and **2** facilitate inward-translocation (flip) of the fluorescently labeled phosphatidylcholine probe, 1-palmitoyl-2-(*N*-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]aminohexanoyl)-*sn*-glycero-3-phosphocholine (PC-NBD).[23]

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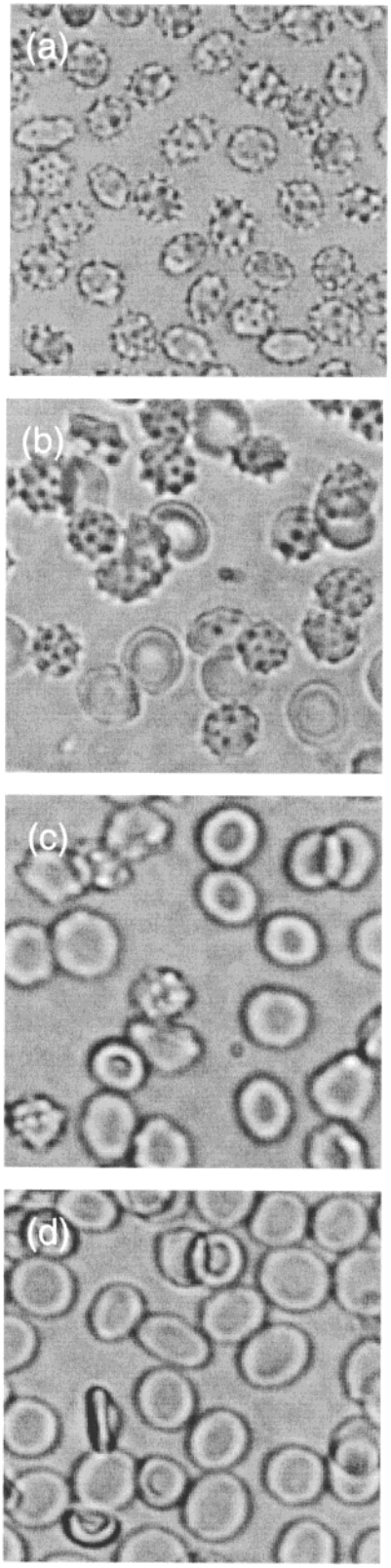

Figure 4. Erythrocyte shapes. The echinocytes shown in panel a were formed immediately after addition of DLPC (35 μM). Separate batches of the echinocytes were incubated for 2 h at 37 °C with 35 μM of (b) ester **3**, (c) amide **2**, and (d) sulfonamide **1**.

The inward-translocation assay was initiated by treating a population of erythrocytes with a small aliquot of PC-NBD (1% of total phospholipid). The probe molecules that did not insert into the outer monolayer of the erythrocyte membrane were removed by washing. Subsequent translocation of the probe to the inner monolayer of the erythrocyte membrane was measured by removing a small sample of cells at periodic intervals and using sodium dithionite to chemically quench the probe remaining in the outer monolayer.[23,24] To ensure that dithionite did not enter the erythrocytes and quench the probe on the inside, it was necessary to inhibit the band 3 anion exchanger protein by pretreating the cells with 4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS).[25,26] The data in Figure 7 clearly show that sulfonamide **1** and amide **2** significantly facilitate inward-translocation of the PC-NBD probe as compared to ester **3** or a background control.

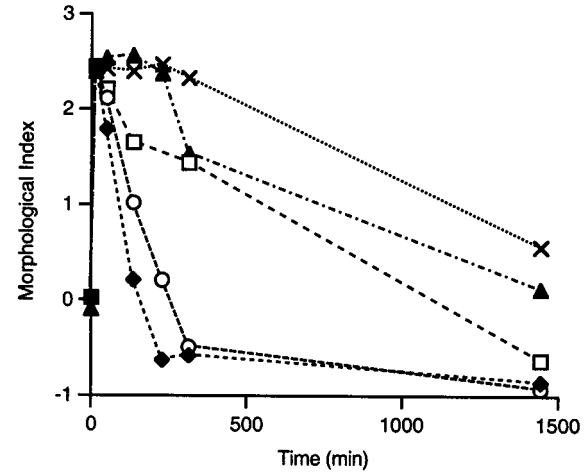

Figure 5. Change in morphological index. Erythrocyte were converted into echinocytes by addition of DLPC (35 μM) at $t = 0$, then at $t = 15$ min separate batches were treated with 35 μM of sulfonamide **1** (filled diamonds), amide **2** (open circles), ester **3** (open squares), chlorpromazine (filled triangles), and no addition (crosses) at pH 7.4 and $T =$ 37 °C. Each point is the average of three independent runs, with an uncertainty of ±0.3 morphological index units.

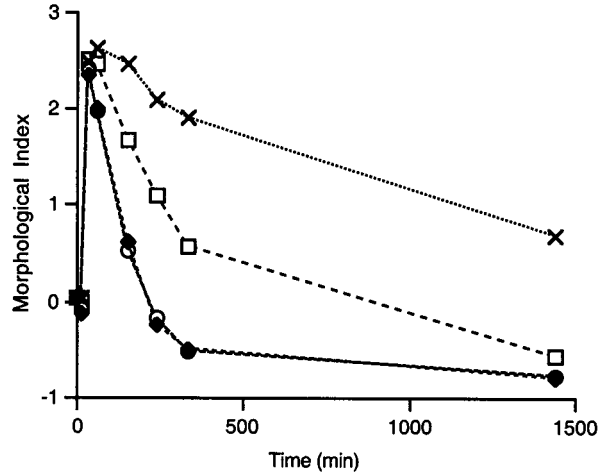

Figure 6. Change in morphological index. Separate batches of erythrocytes were treated at $t = 0$ with 35 μM of sulfonamide **1** (filled diamonds), amide **2** (open circles), ester **3** (open squares), and no addition (crosses), then at $t = 15$ min DLPC (35 μM) was added at pH 7.4 and $T = 37$ °C. Each point is the average of three independent runs, with an uncertainty of ±0.2 morphological index units.

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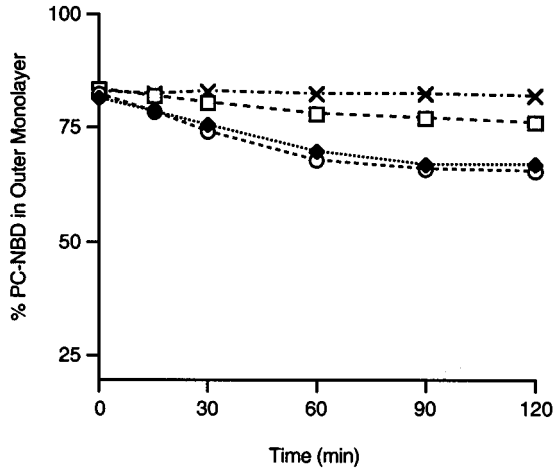

Figure 7. Changes in the percent PC-NBD in the outer monolayer of the erythrocyte membrane at 37 °C and pH 7.4. Inward translocation induced at $t = 0$ by adding 35 μM of sulfonamide **1** (filled diamonds), amide **2** (open circles), ester **3** (open squares), and no addition (crosses) to erythrocytes with PC-NBD (1% of total phospholipid) already inserted into the outer monolayer. Note that at $t = 0$ about 18% of the PC-NBD has already migrated to the inner monolayer due to the preliminary labeling and washing procedures. Each point is the average of three independent runs, with an uncertainty of ±2% units.

Discussion

The pK_a* values of the protonated forms of compounds **1**, **2**, and **3** are 4.5, 5.2, and 4.2, respectively. At neutral pH, it is the free base that binds to the polar membrane surface and subsequently partitions into the lipophilic interior. NMR binding studies in $CDCl_3$ confirm that in a nonpolar environment the free base of sulfonamide **1** forms a 1:1 hydrogen-bonded complex with the phosphocholine headgroup (Scheme 4). As expected, the less acidic amide **2** binds more weakly and the control ester **3** has essentially no binding affinity.[14] This order of binding affinities matches the relative abilities to facilitate phosphatidylcholine translocation through vesicle membranes (i.e. **1** ≫ **2** > **3**).[8]

Compared to a vesicle membrane, a biological membrane is thicker and structurally much more complicated. The inner surface of an erythrocyte is supported by a network of skeletal proteins and extending from the outer surface is the glycocalyx, a mosaic of glycoproteins and glycolipids. Exogenous cationic amphiphiles are known to diffuse through the erythrocyte membrane and accumulate in the anionic, inner monolayer, thus producing stomatocytes.[25−29] Depending on the amphiphile's structure this process may occur over a period of minutes to hours.[30] Certain amphiphilic amines such as chlorpromazine (a phenothiazine drug used in the treatment of schizophrenia) produce stomatocytes within a few minutes. The chlorpromazine rapidly diffuses through the cell membrane and at concentrations below 0.1 mM slightly scrambles the distribution of bilayer phospholipids.[31−33] The scrambling mechanism is not known but one untested suggestion is that the chlorpromazine transiently disrupts the membrane by promoting nonbilayer structures.[30,32] After this sudden, initial perturbation of the membrane, the chlorpromazine has no further effect on the rate of phosphatidylcholine transmembrane diffusion.[20,32]

By comparison, all three translocase candidates, **1**−**3**, induce similarly low amounts of stomatocytosis over 5 h (Figure 3). Involvement of the endogenous aminophospholipid flippase is ruled out because the same results are obtained in the presence of vanadate, a known aminophospholipid flippase inhibitor.[18,19] Thus, it appears that these tertiary amines slowly diffuse through the erythrocyte membrane and intercalate in the inner monolayer. Compared to control ester **3**, sulfonamide **1** and amide **2** are much more effective at converting phosphatidylcholine-induced echinocytes back to disocytes (Figures 4−6). This effect is achieved in two ways: (a) Like other stomatocyte-forming amphiphiles,[20,22] compounds **1**−**3** selectively intercalate and expand the membrane's inner monolayer. (b) The fluorescent PC-NBD translocation assay (Figure 7) shows that **1** and **2** also have a special ability to promote inward phosphatidylcholine translocation. The translocation mechanism can be viewed in the following way.[33] The free bases of sulfonamide **1** and amide **2** form hydrogen-bonded complexes with the zwitterionic phosphatidylchloline headgroups in the membrane outer monolayer (Scheme 4), and the complexes diffuse through the lipophilic interior of the membrane. The inner monolayer is anionic (it is enriched with phosphatidylserine which carries a net negative charge) and accumulates the anion-binding synthetic translocases. In simple vesicle systems, sulfonamide **1** is a much better phosphatidylcholine translocator than amide **2**, but in structurally more complex erythrocyte cell membranes they have similar activity. The reason for this is not clear but one possibility is that the translocation ability of sulfonamide **1** in cell membranes is diminished because a significant fraction of **1** is sequestered from the cell membrane surface by the anionic residues on the glycocalyx.

Summary

In this paper we have shown that synthetic, anion receptors, **1** and **2**, are able to facilitate inward phosphatidylcholine translocation across erythrocyte membranes. Thus, **1** and **2** function as low molecular-weight, synthetic phosphatidylcholine translocases.[4] More specifically they should be classified as synthetic scramblases since they should promote transmembrane diffusion in either direction. In terms of biological activity, it will be interesting and potentially quite useful to develop synthetic translocases for the aminophospholipids, phosphatidylserine and phosphatidylethanolamine, because their presence on the cell surface is known to regulate a range of important dynamic and signal transduction processes.[1−3] With this goal in mind, second generation translocases are currently under investigation and the results will be reported shortly.

Experimental Section

All lipids were purchased from Avanti Polar Lipids, Inc. 4,4′-Diisothiocyanatostilbene-2,2′-disulfonate (DIDS) and sodium orthovanadate were purchased from Aldrich.

Synthesis of Translocases. Translocases **1**−**3** were prepared using the literature method of Valiyaveettil.[14] Sulfonamide **1** was purified by column chromatography on silica (100% $CHCl_3$ → 95:5 $CHCl_3$/MeOH) and obtained in 88% yield (mp 40−43 °C). ^{1}H NMR (300

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MHz, $CDCl_3$) δ 7.82 (d, 6H), 7.32 (d, 6H), 5.64 (t, 3H), 2.97 (q, 6H), 2.54 (t, 6H), 2.42 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 143.1, 136.7, 129.6, 127.0, 53.9, 40.7, 21.3. MS (FAB^+) *m*/*z* 609 $[M + H]^+$. Amide **2** was purified by column chromatography on silica (90:9:1 $CHCl_3$/MeOH/H_2O) and obtained in 90% yield (mp 162−166 °C). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.49 (d, 6H), 7.03 (t, 3H), 6.84 (d, 6H), 3.56 (q, 6H), 2.73 (t, 6H), 2.28 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.2, 141.3, 131.1, 128.8, 127.3, 53.7, 37.8, 21.4. MS (FAB^+) *m*/*z* 501 $[M + H]^+$. Ester **3** was purified by column chromatography on silica (99:1 $CHCl_3$/MeOH) and obtained in 66% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.87 (d, 6H), 7.16 (d, 6H), 4.41 (t, 6H), 3.08 (t, 6H), 2.37 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.8, 143.7, 129.8, 129.2, 127.5, 63.2, 53.6, 21.8. MS (FAB^+) *m*/*z* 504 $[M + H]^+$.

^{1}H NMR Titrations. Translocases, **1**−**3**, were titrated with POPC. Three different NMR tubes each contained a solution of translocase in $CDCl_3$ (5 mM, 750 μL). Small aliquots of POPC stock solution (0.375 M) were added, followed by the acquisition of a ^{1}H NMR spectrum. Concentrations and equivalents were adjusted to give the optimum change in Weber *p* values (0.2−0.8).[34] Titration isotherms were generated for the NH or NCH_2 protons that exhibited significant shifts. Fitting the data to a 1:1 binding model using an iterative curve-fitting method yielded association constants and maximum change in chemical shift.[13]

Erythrocyte Preparation. Blood was obtained from a single healthy donor by venipuncture and treated with EDTA solution (dipotassium salt). Erythrocytes were isolated by centrifugation at 7900 rpm for 5 min, and subsequent washing three times with 4 volumes of ice cold, 150 mM NaCl and once with 4 volumes of an ice cold solution of 138 mM NaCl, 5 mM KCl, 6.1 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, 1 mM $MgSO_4$, and 5 mM glucose at pH 7.4 (NaCl/P_i). The cells were used the same day. All incubations were carried out in NaCl/P_i buffer at 37° C, ∼20% hematocrit in capped plastic tubes unless otherwise stated.

Cell Morphology Assay. Echinocytes were formed by addition of DLPC (35 μM) to normal erythrocytes, followed by a 15 min incubation. Small aliquots of ethanolic solutions of **1**, **2**, **3**, or chlorpromazine (35 μM final concentration) were added to separate batches of the crenated cells. Incubations were carried out for 24 h, with samples periodically removed for morphology assessment. Control experiments were also conducted in which only **1**, **2**, **3**, or chlorpromazine was added to normal erythrocytes at the same concentrations. A second morphology experiment reversed the addition order. First, the cells were incubated with 35 μM of **1**, **2**, or **3** for 15 min. Then, DLPC (35 μM) was added and the experiment continued as described above.

Cell morphology was assessed by light microscopy and graded on the scale of −4 to +5 based on the nomenclature of Bessis.[9,17] The average score of 100 erythrocytes was taken as the morphological index. The scoring was done blind, that is, the worker assigning the scores was unaware of the treatment that each cell population had undergone. A 5.0 μL aliquot of cells was fixed in 50 μL of 0.5% glutaraldehyde in ice-cold 150 mM NaCl solution. A 1.0 μL aliquot of this suspension was viewed under a bright field microscope (Nikon Eclipse e600, 100× DIC H objective) using plastic slides and coverslips; images were acquired using a digital camera. For the vanadate inhibition experiments, the erythrocytes were incubated for 90 min in NaCl/P_i buffer containing sodium orthovanadate (30 μM) prior to DLPC addition.

Inward PC-NBD Translocation Assay.[25] Excitation was set at 470 nm, and 1-palmitoyl-2-(*N*-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino-hexanoyl)-*sn*-glycero-3-phosphocholine (PC-NBD) fluorescence emission was measured at 530 nm using a 515 nm filter. Erythrocytes were incubated for 30 min in NaCl/P_i buffer containing 50 μM DIDS. The cells were washed twice with NaCl/P_i buffer before addition of PC-NBD (20 μM, 1% of total phospholipid) at 0 °C and standing for 15 min. Probe not inserted into the membrane was removed via two NaCl/P_i washings. Ethanolic solutions of **1**, **2**, or **3** (40 μM) were added. The resulting solutions were incubated for 2 h. Periodically, 50 μL samples were removed, diluted in 2 mL of NaCl/P_i, and assayed for inward translocation. The assay consisted of a dithionite injection (88 mM) at $t = 50$ s and a Triton X-100 injection (0.5%) at $t = 200$ s. Upon addition of dithionite the PC-NBD emission intensity immediately spikes, apparently due to dithionite-induced weakening of a hemoglobin inner filter effect.[35] The percentage of probe in the outer monolayer was calculated according to the following equation, % exo PC-NBD $= [(F_i - F_f)/F_i] \times 100$, where F_i and F_f are the intensities just after the addition of dithionite and just before the addition of Triton X-100, respectively.

Critical Micelle Concentration Determination.[15] Excitation was set at 480 nm, and fluorescence emission was measured from 500 to 650 nm with an open filter. A series of 3 mL samples of varying translocase concentration were prepared in 7.3 μM rhodamine 6G chloride, 5 mM of *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer, and 100 mM NaCl at pH 7.4. The samples were incubated at room temperature for 30 min in the dark before obtaining the fluorescence spectrum. Emission was recorded at 550 nm as a function of the translocase concentration. Above the CMC, a decrease is observed in the fluorescence intensity. The data points form two distinct lines, and the intersection of the lines represents the CMC value. A value of 0.13 mg/mL was obtained for Triton X-100, which is close to the literature value.[16]

Hemolysis Assay.[36] Absorbance was monitored from 600 to 300 nm. A series of 1 mL samples was prepared: 0% hemolysis (only NaCl/P_i buffer), 100% hemolysis (only H_2O), **1** (35 μM in buffer), **2** (35 μM in buffer), and **3** (35 μM in buffer). To each, 100 μL of packed red blood cells was added (∼10% hematocrit). Samples were incubated at 37 °C for varying time periods before removal of 100 μL aliquots for hemolysis analysis. The aliquots were centrifuged for 10 min at 1000 rpm, and the supernatant was diluted to 1 mL with NaCl/P_i buffer before recording the absorbance of the hemoglobin in solution (*A* at 414 nm). The percent hemolysis was calculated according to the following equation, % hemolysis $= 100 - [(A_{100\%} - A/A_{100\%} - A_{0\%}) \times 100]$.

pK_a Determination.[11] pK_a was determined by potentiometric titration in 9:1 methanol−water and is thus referred to as pK_a*. Because the pH meter was calibrated using aqueous standards, a mixed solvent correction factor of 0.5 was added to the reading on the pH meter.[37] A small aliquot of HCl (2.00 mM) was added to compound **1**, **2**, or **3** (1.00 mM) in 9:1 methanol−water and the resulting solution then titrated with standardized NaOH solution. In each case, a definite equivalence point was observed and used to calculate the pK_a* value.

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