Detection of Nonideal Mixing of Phospholipids in Fluid Bilayers

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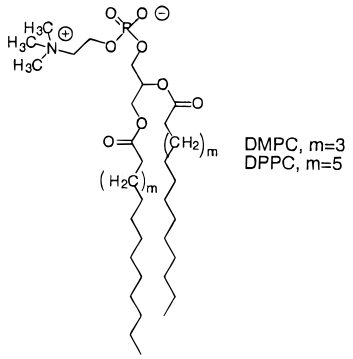
Abstract: A synthetic ionophore (**1**) has been prepared via condensation of 23,24-bisnor-5-cholenic acid with both amino groups of 1,17-diamino-3,6,9,12,15-pentaoxaheptadecane and used to probe the mixing behavior of a series of unsaturated phosphocholines (PCs) in the fluid bilayer state. Specifically, the ability of **1** to promote the transport of Na^+ across membranes made from equimolar mixtures of "long" and "short" PCs has been compared with that in analogous membranes derived from single-component PCs of intermediate length. Relative ionophoric activities have been measured by use of $^{23}Na^+$ NMR spectroscopy. The results of this study provide compelling evidence that a hydrophobic mismatch of four methylene groups can lead to nonideal mixing of phosphocholines in fluid bilayers.

Introduction

Clarification of the mixing behavior of phospholipids in the fluid bilayer state has proven to be a formidable challenge, and one that has considerable biological relevance.[1−13] In particular, the time-averaged lateral distribution of phospholipids within biological membranes is presumed to play an important role in many cellular processes that are essential to the living state; e.g., membrane fusion events, cell surface recognition, membrane protein activity, etc.[13,14] At present, however, the two-dimensional structures of all biological membranes remain virtually unknown. A major difficulty has been the absence of experimental methods that are directly applicable to fluid bilayers. It is noteworthy in this regard that even for the simplest of model systems, conclusions concerning lipid mixing have sometimes proven controversial. In one previous work, for example, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) were judged to be completely miscible in the fluid bilayer state based on phase diagram analyses.[15] In a separate investigation that focused on this same DPPC/DMPC system, complete immiscibility was inferred, based on quick-freeze differential scanning calorimetry results.[16−18]



Recently, a variety of chemical cross-linking approaches have been introduced for studying phospholipid mixing. In two related methods, phospholipid dimerizations have been carried out between nearest-neighbors by use of irreversible photochemical or condensation reactions.[19,20] Analysis of the resulting dimer distributions then provides an indication as to whether the lipids are randomly mixed. Nearest-neighbor recognition (NNR) analysis, a technique that we have introduced ourselves, bears a close resemblance to these chemical cross-linking methods.[21] In essence, the NNR method makes use of reversible disulfide exchange reactions between thiol-functionalized phospholipid derivatives. A unique and important feature of the NNR method is that it provides direct thermodynamic insight into nearest-neighbor interactions. Specifically, it quantifies the thermodynamic preference for one phospholipid to become a nearest-neighbor of another. A principal shortcoming of all of these "dimer" approaches, however, is that they rely upon the use of phospholipid derivatives.

In this paper, we describe the synthesis of a novel bis-sterol/polyether conjugate, **1**, which functions as a synthetic ionophore.

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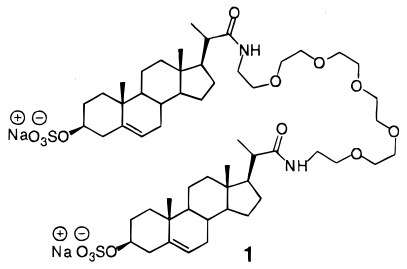
We also show how **1** can provide fundamental insight into the effects of hydrophobic mismatch on the mixing behavior of *underivatized* phosphocholines in fluid bilayers.[22] Specifically, we show how the ionophoric activity of **1** can be used to provide compelling evidence that a hydrophobic mismatch of four methylene groups can lead to nonideal mixing of phosphocholines within the fluid bilayer state.



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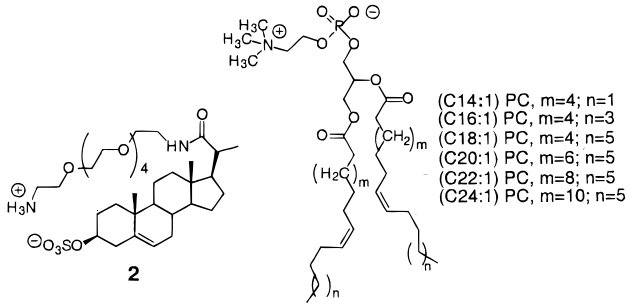
Rationale for the Use of Ionophoric Activity To Probe Phospholipid Mixing. In previous studies, we have shown that a synthetic ionophore derived from 23,24-bisnor-5-cholenic acid and 1,17-diamino-3,6,9,12,15-pentaoxaheptadecane (i.e., **2**) is active in transporting Na^+ across bilayers made from 1,2-dimyristoleoyl-*sn*-glycero-3-phosphocholine [(C14:1) PC].[23] We have also shown that the activity of **2** is *highly sensitive to*



2

membrane thickness. Thus, in thicker bilayers [i.e., those made from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, (C18:1) PC] the activity of **2** decreased by more than a factor of 300, compared with (C14:1) PC. Moreover, the dependence of ionophoric activity on the bilayer thickness is strongly nonlinear; i.e., the activity in (C16:1)PC [1,2-dipalmitoleoyl-*sn*-glycero-3-phosphocholine] is significantly lower than the average of the activity in (C14:1)PC and (C18:1)PC.

At that time, these observations suggested to us that **2** might be a useful tool for investigating the influence of chain mismatch on the mixing properties of phospholipids.[24] Specifically, we reasoned that a comparison of the activity of **2** in a membrane composed of an equimolar mixture of a "short" and a "long" phospholipid versus a membrane made from a single phospholipid of "intermediate" length could provide insight into the mixing behavior of the former. In principle, perfect mixing of the short and the long phospholipid should produce an average membrane thickness and microenvironment that is essentially the same as that formed from the intermediate phospholipid. In such a case, the activity of **2** in both membrane types would be expected to be the same. If the short and long phospholipids were nonideally mixed, however, then the observed ionophoric activities *are likely to be different*. This would depend largely on the distribution of **2** throughout the thick and thin regions of the membrane. For example, if **2** were partitioned homogeneously throughout the thick and thin regions, one might expect to observe a *higher* activity. In the limiting case, where there is complete separation of the membrane into thick and thin regions, the ionophoric activity is expected to be the average of the activity in membranes made from the pure long and the pure short lipid. Due to the strong dependency of ionophoric activity on membrane thicknesss, this average would then be expected to be significantly higher than the activity that is found in the pure intermediate lipid.

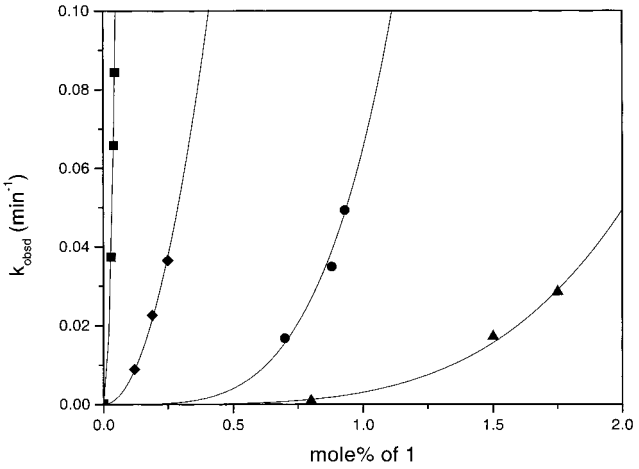


Figure 1. Plot of k_{obsd} versus (mol % of **1**) for the entry of Na^+ into vesicles made from (■) (C14:1) PC, (◆) (C16:1) PC, (●) (C18:1) PC, and (▲) (C20:1) PC at 35 °C. The solid lines are best-fit curves based on eq 1, where $n = 2.0 \pm 0.02$, 1.9 ± 0.1, 4.0 ± 0.2, and 3.7 ± 0.2, respectively.

In contrast, if the ionophore would strongly favor thick regions, then the observed activity could be lower. Thus, *a difference in activity for* **2** *between the two membrane types would constitute strong evidence for nonideal mixing*.

Although we were intrigued with this concept, the activity of **2** proved to be too low to examine a broad range of phospholipids. A recent discovery, however, has now allowed us to explore this approach. Specifically, we have found that a related conjugate (i.e., **1**) possesses much higher activity for Na^+ transport [2500 times greater than **2** in (C14:1) PC membranes] and that it also shows higher sensitivity toward bilayer thickness.[25]

Results

Ionophoric Activity in Bilayers Composed of Single Phospholipids. Before investigating the ionophoric properties of **1** in mixed membranes, we first examined its activity in a series of bilayers made from (C14:1) PC, (C16:1) PC, (C18:1) PC, and (C20:1) PC [1,2-dieicosenoyl-*sn*-glycero-3-phosphocholine].[26] Using experimental procedures similar to those

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(25) Recently, a cholic acid-based sterol dimer, which bears a structural relationship to **1**, has been found to possess high ionophoric activity: Kobuke, Y. *Artificial Ion Channels: Mimics of Biological Signal Transduction*; National Institute for Advanced Interdisciplinary Research: Tsukuba, Japan, March 1, 1999; p 34.

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previously described, the rate of Na^+ transport into large unilamellar vesicles (LUVs, 2000 Å diameter) was monitored via $^{23}Na^+$ NMR spectroscopy.[27] In brief, a membrane-impermeable paramagnetic shift reagent is added to the external aqueous solution and the percentage of Na^+ that enters the vesicles is quantified by integration of internal and external $^{23}Na^+$ NMR absorbances, and followed as a function of time.

In the case of the shorter lipids [(C14:1) PC and (C16:1) PC], the observed pseudo-first-order rate constant (k_{obsd}) was found to have a second-order dependency on the mole percent of **1** that was present; with the longer lipids [(C18:1) PC and (C20:1) PC], a fourth order dependency was found (Figure 1). Note that the dependence of the rate constants at a given ionophore concentration on the bilayer thickness is strongly nonlinear.

As noted previously, a nonlinear dependence of transport activity on ionophore concentration provides strong support for a model in which a small fraction of the ionophore is assembled into transport-active aggregates.[23] Specifically, it can be shown that:

$$k_{obsd} = k_o + k_2[\text{monomer}]^n/K \quad (1)$$

where K is the equilibrium constant for dissociation of an assembly of n ionophore molecules into monomers, k_o is the rate constant for ion transport in the absence of ionophore, and k_2 is an intrinsic rate constant.[23] Thus, our data indicate the presence of transport-active *dimers* and *tetramers* in the thinner and thicker membranes, respectively. The apparent shift from a dimer-active to a tetramer-active species, on going to thicker bilayers, can be accounted for by inherent differences in the hydrophobic−hydrophilic balance between the ionophore and the phospholipid. Specifically, as the length of the acyl chains of the phospholipid increases, the mismatch in the hydrophobic−hydrophilic balance between the two molecules also increases, which can result in the formation of a higher ordered aggregate.

Finally, it should be noted that the strong dependence of ionophoric activity on membrane thickness, which is evident for **1**, would not be expected for a carrier mechanism. Rather, it argues strongly that a membrane-spanning structure is responsible for the flow of ions across the membrane.[23] Similar arguments have previously been made for **2**.[23]

Ionophoric Activity in Bilayers Composed of Two Phospholipids. The above data were then used as a basis from which to select concentrations of **1** that would be convenient for monitoring Na^+ transport within a series of mixed membranes. Thus, out of necessity, different concentrations of ionophore were chosen for each series of membranes having different averaged thicknesses. It should be noted, however, that *identical* concentrations of ionophore within each series allow us to make direct comparisons between membranes made from a mixture of long and short phospholipids and those made from single phospholipids.

A summary of our principal findings is presented in Table 1. In brief, the activity of **1** in bilayers made from (C16:1) PC was essentially the same (±10%) as that found in bilayers made from a 1/1 mixture of (C14:1) PC/(C18:1) PC. In contrast, the same hydrophobic mismatch of four methylene units in an equimolar mixture of (C16:1) PC/(C20:1) PC yielded a rate constant that was 1.8 times greater than that of pure (C18:1) PC. When the hydrocarbon mismatch was extended to eight methylenes, using a 1/1 mixture of (C14:1) PC/(C22:1) PC, the rate constant was now 5.9 times larger.

Table 1. Ion Transport Activity of **1** in Fluid PC Bilayers[a]

av chain length	lipid mixture	$\Delta(CH_2)_n$[b] for n =	mol % **1**	$10^2 k_{obsd}$ (min^{-1})
14	C14:1	0	0.22	200[c]
16	C16:1	0	0.22	2.90 (1.0)
	C14:1/C18:1	4	0.22	3.25 (1.1)
17	C16:1/C18:1	2	0.47	3.44 (1.0)
	C14:1/C20:1	6	0.47	8.11 (2.4)
18	C18:1	0	0.63	1.13 (1.0)
	C16:1/C20:1	4	0.63	2.07 (1.8)
	C14:1/C22:1	8	0.63	6.70 (5.9)
	C14:1/C18:1/C22:1	8/0[d]	0.63	3.29 (2.9)
	C14:1/C18:1/C22:1	8/0[e]	0.63	2.38 (2.1)
19	C18:1/C20:1	2	0.75	1.16 (1.0)
	C16:1/C22:1	6	0.75	2.16 (1.9)
	C14:1/C24:1	10	0.75	3.75 (3.2)
20	C20:1	0	1.25	0.74 (1.0)
	C18:1/C22:1	4	1.25	1.47 (2.0)

[a] All transport rates (±10%) were measured at 35 °C. The gel to liquid-crystalline phase transition temperature of the highest melting phospholipid that was used in this study of (C24:1) PC is 24 °C.[26] For convenience, parentheses and PC designations have been omitted; all binary systems were made using an equimolar amount of the two phospholipids. Numbers in parentheses represent relative rates. [b] Hydrocarbon chain-length mismatch. [c] Extrapolated value from the data in Figure 1. [d] 33 mol % of C18:1 was used as a diluent. [e] 50 mol % of C18:1 was used as a diluent.

Examination of k_{obsd} as a function of the mole percent of **1** in a 1/1 mixture of (C14:1) PC/(C22:1) PC revealed *the same fourth-order dependency that was found for bilayers composed of pure* (C18:1) PC (Figure 2).

Incremental dilution of a (C14:1) PC/(C22:1) PC-membrane with (C18:1) PC resulted in a steady decrease in the rate constant approaching that found in pure (C18:1) PC membranes.

Results that were obtained in bilayers having average chain lengths of 17, 19, and 20 carbons showed, qualitatively, the same trend; i.e., within each of these series, an increase in the chain length difference resulted in a significant increase in the observed rate constant (Table 1). It may be noted that rate constants for **1** in membranes made from pure (C22:1) PC, and also pure (C24:1) PC, bilayers have not been included in Table 1. The reason for this is that the rate of Na^+ transport of **1** was too low in these membranes to measure.

Discussion

Detection of Nonideal Mixing. Results that have been obtained with membranes having an average acyl chain length of 18 carbons provide a compelling case for nonideal mixing. Thus, a comparison of the activity of **1** in bilayers formed from (C16:1) PC/(C20:1) PC with that found in pure (C18:1) PC membranes reveals a *significant difference*, i.e., the activity being greater in the former. Extending the chain length mismatch to eight methylene units, by use of a (C14:1) PC/(C22:1) PC membrane, resulted in an even higher observed rate constant. These results imply that both (C16:1) PC/(C20:1) PC and (C14:1) PC/(C22:1) PC are nonideally mixed.

The decrease in the rate constant for Na^+ transport, which accompanies dilution with (C18:1) PC, provides further support for nonideal mixing in the absence of this diluent. Specifically, (C18:1) PC is likely to function as a mixing agent, which helps to produce a more homogeneous membrane.[28]

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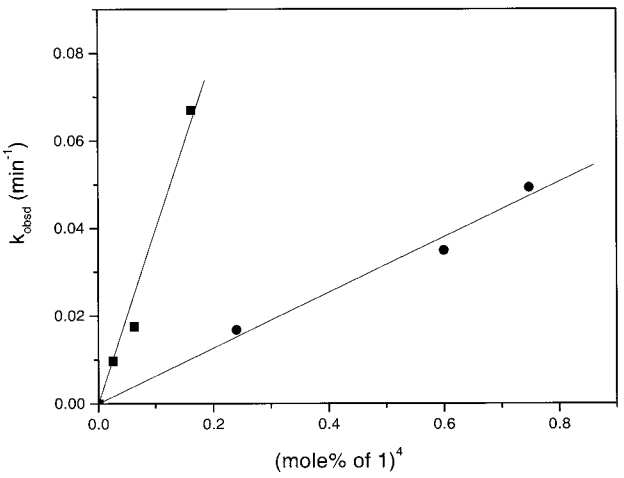

Figure 2. Plot of k_{obsd} versus (mol % of **1**)^4 for the entry of Na^+ into vesicles made from (●) (C18:1) PC and (■) a 1/1 mixture of (C14:1) PC/(C22:1) PC at 35 °C.

The fact that the rate of Na^+ transport across (C14:1) PC/(C22:1) PC bilayers exhibits a fourth-order dependency on the mole percent of **1** (which is the same as that found for the pure (C18:1) PC membrane) rules out the possibility that the main pathway for ion transport is occurring through "domains" of pure (C14:1) PC, since a second-order dependency should have been observed, as is shown in Figure 1. Comparison of k_{obsd} in bilayers of (C14:1) PC/(C20:1) PC, (C14:1) PC/(C22:1) PC, and (C14:1) PC/(C24:1) PC (i.e., membranes made from phospholipids having an average acyl chain length of 17, 18, and 19 carbons) provides further support for this conclusion. Thus, if Na^+ transport were occurring through domains of pure (C14:1) PC, then an increase in the length of the co-lipid would be expected to increase the importance of such domains, i.e., the rate constant should increase. What is observed, however, is a decrease in the rate constant, despite the presence of increasing mole percentages of ionophore (Table 1). This decrease in the rate constant is undoubtedly due to the effects of membrane thickness on ionophoric activity.

These results are suggestive of a situation in which the longer phospholipids have a tendency to self-associate, leaving behind portions of the membrane that are *partially* depleted in this lipid, and that are, therefore, relatively enriched in the shorter lipid and, consequently, thinner than average.

Finally, the significant differences in the rate constants observed within each series of bilayers, having an average acyl chain length of 17, 19, and 20 carbons, also imply the existence of nonideal mixing.

Results that have been obtained with membranes made from (C14:1) PC and (C18:1) PC do not allow for unambiguous conclusions to be drawn in terms of their mixing behavior. Most likely, the appearance of a rate constant that is essentially the same as that which has been found for the intermediate (C16:1 PC)-based membrane is a consequence of ideal mixing of the two lipids. However, an alternate situation is also possible. In particular, if the lipids were nonideally mixed and the ionophore strongly favored the thicker regions of the membrane, then the rate constant could, by coincidence, be exactly the same as that found in pure (C16:1) PC. Thus, while we suspect that (C14:1) PC and (C18:1) PC are ideally miscible, our data do not permit us to draw clear conclusions in this case.

In our view, the results reported herein provide the strongest evidence to date that a mismatch of hydrocarbon chain length of four methylene groups between *underivatized* phosphocholines can lead to nonideal mixing in the fluid bilayer state. Although the possibility exists that the presence of **1** influences the mixing behavior of these lipids, the fact that similar results were obtained with different ionophore concentrations (Table 1) implies that such effects are negligible. Efforts aimed at extending this ionophoric approach to the study of lipid mixing are now in progress.

Experimental Section

***O*-[*N*-1,2,3-Benzotriazin-4(3*H*)one]-yl-23,24-bisnor-5-cholen-3-*β*-ol-ate**. To a magnetically stirred suspension of 0.470 g of 23,23-bisnor-5-cholenic acid-3-ol (1.36 mmol) in 5 mL of anhydrous THF plus 5 mL of CH_2Cl_2 was added dropwise a solution of 0.279 g of dicyclohexylcarbodiimide (1.45 mmol) in 10 mL of CH_2Cl_2 at 10 °C. After addition was completed (ca. 10 min), 0.240 g of 3-hydroxy-1,2,3-benzotriazin-4(3*H*)-one (1.47 mmol) was added and the mixture was allowed to stir at room temperature for 24 h. Subsequent removal of solid via filtration and concentration under reduced pressure afforded 0.340 g of crude product, which was purified by preparative TLC [silica, $CHCl_3$/acetone (6:1,v/v)] to give 0.211 g (42.9%) of the title compound: R_f 0.70; ^{1}H NMR (360 MHz, $CDCl_3$) δ 8.36 (d, 1H), 8.20 (d, 1H), 7.96 (t, 1H), 7.82 (t, 1H), 5.35 (m, 1H), 3.49 (m, 1H), 2.88 (m, 1H), 1.03−2.29 (m, 26H), 0.81 (s, 3H).

***N*$_1$,*N*$_2$-Bis-1,17-(3,6,9,12,15-pentaoxaheptadecanediyl)-23,23-bisnor-5-cholen-3*β*-ol-amide.** To a stirred solution of 0.110 g of *O*-[*N*-1,2,3-benzotriazin-4(3*H*)one]-yl-23,24-bisnor-5-cholen-3*β*-ol-ate (0.224 mmol) in 1.0 mL of anhydrous DMF was added a solution of 31.3 mg (0.11 mmol) of 1,17-diamino-3,6,9,12,15-pentaoxaheptadecane in 0.50 mL of anhydrous DMF containing 0.1 mL of *N*,*N*-diisopropylethylamine. The mixture was first stirred at room temperature for 16 h and then added dropwise to 20 mL of 10% Na_2CO_3/H_2O. The precipitate that was formed was separated, washed with 20 mL of H_2O, dried (1 Torr, 23 °C, 2 h), and purified by a combination of preparative thin layer and column chromatrography [silica, $CHCl_3$/MeOH, 10:1 (v/v)] to give 59.8 mg (58%) of title conjugate: R_f 0.48; ^{1}H NMR (360 MHz, CD_3OD) δ 5.29 (d, 2H), 3.62 (m, 16H), 3.49 (m, 4H), 3.35 (m, 2H), 3.28 (m, 4H), 0.98−2.21 (m, 54H), 0.69 (s, 6H).

***N*$_1$,*N*$_2$-Bis-1,17-(3,6,9,12,15-pentaoxaheptadecanediyl)-23,23-bisnor-5-cholen-3*β*-sulfate-amide, Disodium Salt (1).** A heterogeneous mixture composed of 37.8 mg (0.0425 mmol) of N_1,N_2-bis-1,17-(3,6,9,12,15-pentaoxaheptadecanediyl) -23,23-bisnor-5-cholen-3β-ol-amide, 105 mg (0.66 mmol) of Py·SO_3, and 2 mL of $CHCl_3$ was stirred in a closed flask at room temperature for 48 h. The solution was then separated by filtration, and the solid was washed twice with 5 mL of $CHCl_3$. The combined organic solution was then concentrated under reduced pressure to give 50.0 mg of solid which was redissolved in 1 mL of MeOH. The resulting solution was added dropwise to 6 mL of 10% Na_2CO_3, and the solid that was formed was separated and washed twice with 3 mL of H_2O to give 46 mg of white powder. This crude product was purified by flash column chromatography [silica, $CHCl_3$/CH_3OH/H_2O, 65/25/3 (v/v/v)] to give 36 mg (78%) of the title compound: R_f 0.32; ^{1}H NMR (360 MHz, CD_3OD) δ 5.37 (d, 2H), 4.12 (m, 1H), 3.63 (m, 16H), 3.53 (m, 4H), 3.29 (m, 4H), 1.03−2.50 (m, 54H), 0.75 (s, 6H); HRMS for ($C_{56}H_{90}N_2O_{15}S_2Na$) $[M^{2-} + Na^+]^-$ calcd 1117.5680, found 1117.5720.

Vesicle Formation. Typically, 6.57×10^{-2} mmol of lipid (25 mg/mL of solution in chloroform, Avanti Polar Lipids) was transferred to a Pyrex test tube. The required amount of ionophore was added from a stock solution in methanol. While rotating the tube, the organic solvents were evaporated using a stream of nitrogen, resulting in a thin film of lipid/ionophore. The last traces of solvent were then removed under reduced pressure (25°, 12 h, <0.2 Torr). To the dried film was added 1.0 mL of a 150 mM LiCl solution that was 10% in D_2O and 90% in H_2O. The mixture was then vortex mixed for 30 s and incubated for 5 min at room temperature, followed by another 30 s of vortex mixing and a 20 min incubation period. The dispersion was then subjected to 5 freeze/thaw cycles (−196 °C/60 °C), followed by sequential extrusion through a 400 nm Nuclepore membrane (10 times) and a 200 nm membrane (10 times). After extrusion, the sample was

incubated for 1 h at room temperature. When (C24:1) PC (T_m = 24 °C) was used, vortexing, incubation, and extrusion were performed at 40 °C.

Na^+ Transport Measurements. In a 10 mm quartz NMR tube was mixed 1.50 mL of a 150 mM NaCl solution 10% in D_2O and 90% in H_2O with 0.200 mL of a shift reagent solution (10 mM $DyCl_3$, 30 mM $Na_5P_3O_{10}$ in 10% D_2O plus 90% H_2O). To this solution was added 0.750 mL of a given vesicle dispersion. The resulting dispersion was then vortex mixed for 10 s. When using (C24:1) PC, all solutions were preheated to 40 °C. $^{23}Na^+$ peak areas were calculated by use of a fitting procedure that was available on the Bruker software. Pseudo-first-order rate constants were calculated from the change in the percentage of encapsulated Na^+ as a function of time, using a curve-fitting procedure.

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