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COMMUNICATIONS

Published on Web 10/06/2006

Sodium Ion Internalized within Phospholipid Membranes

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Ion passage through phospholipid membranes is a venerable[1−6] and honored[7,8] area of bio-organic/biophysical chemistry. Briefly, ions can pass through phospholipid bilayers via channel compounds (e.g., gramicidin A, amphotericin B, and blepharismin 1[9]) or via ion carriers (e.g., valinomycin and monensin). Transport occurs either along diffusion gradients ("passive") or against diffusion gradients ("active"), the latter requiring energy input. In previous work, mobile ions were present on both sides of the membrane, but they have never been detected, that we know of, within the membrane itself. This is not surprising because the interior of a phospholipid membrane is comprised of hydrocarbon chains—hardly a propitious environment for ionic species. Indeed, it is this very environment that provides the barrier to ion flux. In the present communication, we provide ^{23}Na NMR evidence that Na^+ can locate itself inside the actual phospholipid bilayer. To achieve such behavior, we used a vesicular membrane composed of a conventional phospholipid (1-palmitoyl-2-oleoyl phosphatidylcholine or POPC) admixed with a lesser amount of a "designer" phospholipid that possesses ester groups along its normally all-hydrocarbon chains. We will demonstrate that the ester groups create membrane "pockets" in which sodium ions reside.

Seven ester-modified phospholipids were synthesized (Figure 1) according to multistep routes given in the Supporting Information. Suffice it to mention here that each lipid was twice chromatographed to give, in low overall yields, pure compounds that had the expected ^{1}H and ^{13}C NMR's and high-resolution mass spectra (ESI). Among the seven lipids in Figure 1, the total methylene count per hydrocarbon chain ranges from 8 to 17. Terminal segments (an important variable as it turns out) vary from 2 to 10 carbons per chain. Five of our phospholipids each possess two ester groups per chain, whereas two phospholipids have chains with only a single ester group.

Vesicles (60−75 nm in diameter as determined by DLS) were prepared by hydrating a thin film of a POPC/ester-lipid mixture in 0.15 M NaCl/D_2O and then extruding the resulting suspension (30 mM lipid) 19 times through a 100 nm polycarbonate filter. Sodium flux was monitored using a procedure pioneered by F. G. Riddell in the mid-1980s:[10] To a vesicle suspension (0.75 mL) was added 10 mg of thulium shift reagent, Tm[DOPTP]$^{5-}$.[11] Thereupon, the intense ^{23}Na NMR signal of sodium external to the vesicles was shifted downfield by about 30 ppm, whereas the weak signal of Na^+ inside the vesicle cavities remained unaltered. The signal of internal Na^+ is weak owing to the small "capture volume" of the dilute vesicle system. We used an INOVA 600 MHz NMR operating at 158 MHz (21−22 °C, 6000 scans).

Four of the seven ester-lipids in Figure 1 (**A**, **C**, **D**, **G**), admixed with POPC at 30 mol %, lack the inner sodium peak shown by a pure POPC system (Figure 2). Inside/outside Na^+ exchange across the vesicle bilayer must be so fast that the inner Na^+ signal melds into the dominant outer Na^+ signal. Defects are apparently created by the four ester-lipids, allowing fast Na^+ exchange on the NMR time scale. In contrast, 30 mol % of **B**, **E**, and **F** has no effect on

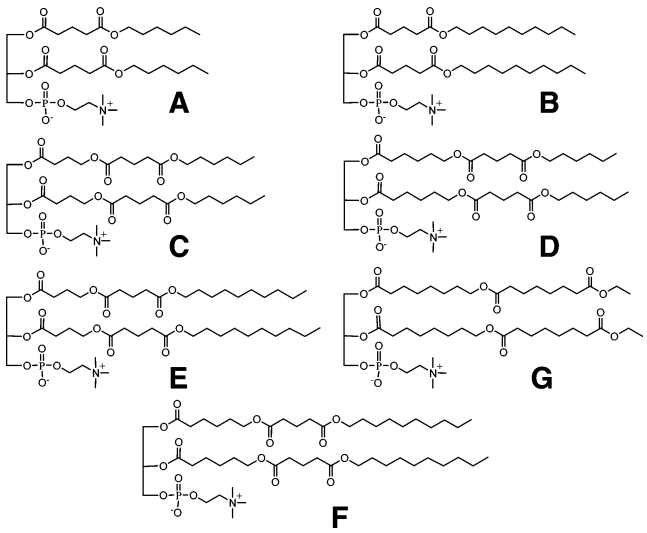

Figure 1. Structures of seven ester-modified phospholipids.

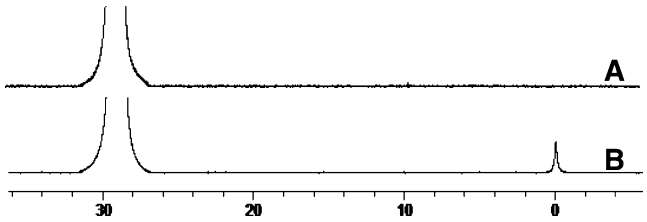

Figure 2. ^{23}Na NMR spectra of vesicular **A** and **B** (30 mol %) admixed with POPC (0.15 M sodium chloride in the presence of Tm[DOTP]$^{5-}$). The spectrum of **B** is identical to the spectrum of the pure POPC system.

the ^{23}Na spectra relative to those from pure POPC vesicles (Figure 2). Both ^{23}Na signals of **B**, **E**, and **F** systems have normal shifts and line widths even at the high 30 mol % concentration and after 2 days of aging in the presence of the shift reagent. Since the three "normal-acting" ester-lipids are the only ones with long terminal segments (i.e., 10 hydrocarbon carbons), we can conclude that short terminal segments are critical for promoting rapid Na^+ interchange. Momentarily we will explain why.

A detailed examination of ester-lipid **G** is particularly illuminating. As mentioned, the baseline near 0 ppm at 30 mol % **G** is featureless, but at lower **G** levels (5−27.5 mol %), an internal Na^+ signal (Figure 3a) is visible. More interesting, a new broad peak develops at 22.5 mol % **G** but disappears at 25 mol %. The mystery of this third ^{23}Na signal was compounded by the discovery that the signal is time-dependent. Thus, all spectra in Figure 3a were taken 30 min after vesicle preparation. If, however, we waited 0.5−13 h for the vesicles to age prior to taking an NMR, a 20 mol % **G** preparation gave spectra shown in Figure 3b. It is seen that within 3 h the single peak splits into two peaks. Although both peaks broaden with time, the "new" peak on the left shifts further downfield by 0.6 ppm over several hours.

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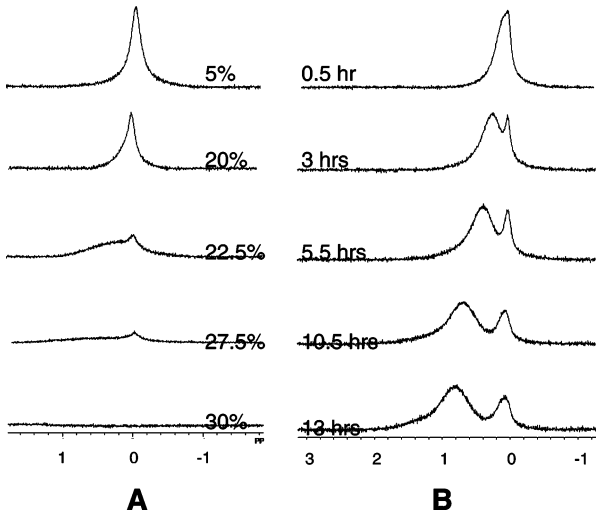

Figure 3. (A) Details of the inner sodium peak region for various mol % of **G** in POPC. Tm[DOTP]$^{5-}$ = 10 mg/0.75 mL. (B) ^{23}Na NMR of 20 mol % of **G** in POPC studied over time. Tm[DOTP]$^{5-}$ = 3.3 mg/0.75 mL.

We estimate from the line width of the higher field signal, representing internal Na^+, that Na^+ flux after 10.5 h is roughly 200 times faster than that occurring with pure POPC vesicles. The same time-dependent double-peak formation was observed using $Dy(PPPi)_2^{7-}$ as the shift reagent, except that the two peaks now appeared downfield from the intense external Na^+ signal.

What is the origin of this extra ^{23}Na signal located slightly downfield from the internal Na^+ signal in Figure 3b? Since one NMR signal with pure POPC vesicles comes from Na^+ in the bulk water and the other NMR signal comes from Na^+ in the vesicular water pools, the third signal in Figure 3b must derive from Na^+ residing in or on the membrane phase. Evidence suggests that this "membrane Na^+" lies within the bilayer as opposed to binding at the vesicle surface: (a) The existence of a membrane Na^+ signal indicates that the ion is not rapidly exchanging on the NMR time scale (in the millisecond regime). It is difficult to imagine Na^+ ions residing at the vesicle surface, or within a surface pocket, that exchange only slowly with excess Na^+ in the water. (b) The chemical shift of the membrane Na^+ (being only slightly downfield from that of internal Na^+) indicates that membrane Na^+ is not in close contact with shift reagent.[12] Membrane-buried Na^+, in contrast to surface-bound Na^+, would have this property (assuming, of course, that shift reagent, with its penta-anionic charge, cannot enter the membrane). The broader line width of membrane Na^+, relative to internal Na^+ (Figure 3b), may result from loss of motional freedom within the membrane pocket.

It remains to explain why the appearance of membrane-buried Na^+ should be time-dependent. A likely rationale is that ester-lipid **G** molecules are initially randomly distributed among the excess POPC molecules in the vesicle bilayer. This is followed by an intra-leaflet migration in which ester-lipid **G** slowly gathers into a domain,[13] thereby creating a membrane "defect" containing Na^+ and water (Figure 4). A need to create opposing domains in the two membrane leaflets, possibly requiring a slow gathering of domain fragments, may impede the process. Simple calculations show that there is roughly one membrane Na^+ per lipid-ester in

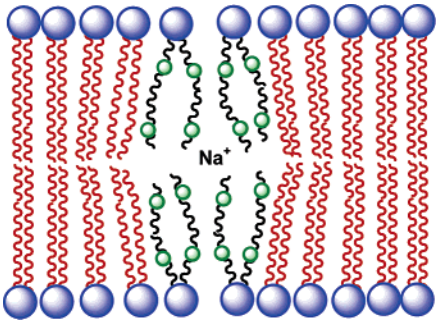

Figure 4. A cross-section of an ester-lipid domain containing a sodium ion and its associated water. The exact location of the ion within the bilayer may vary with time.

pockets where, presumably, Na^+ is coordinated with both water and the ester carbonyl groups.[14] If the domains serve as a conduit for Na^+ flux, then the NMR signal of the inner Na^+ will decrease as the domains form.

When the terminal chain segments are long (as in ester-lipids **B**, **E**, and **F**), the lipids "ideally" intermix with the POPC, domain formation does not occur, and catalyzed flux is not observed. Lipids with short terminal segments (**A**, **C**, **D**, and **G**) are, however, able to migrate and form domains that solubilize Na^+ within the hydrocarbon interior.

We cannot help wondering about unchartered chemistry that is likely concealed within inner sanctums of live membranes.

Acknowledgment. This work was funded by the National Institutes of Health. A.L.G. was supported by a Woodruff Fellowship, and M.E.C. by an ARCS Scholar Award. We appreciate helpful discussions with Dr. Dan Lundberg.

Supporting Information Available: Synthetic routes to the new lipids and their spectral characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) Tm[DOPTP]$^{5-}$ = thulium(III)(1,4,7,10-tetraazacyclododecane-1,4,7,10-tetramethylenephosphonate). Macrocyclics M-155.
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(14) We have also considered the possibility that the double high-field peaks (Figure 3b) arise from a population of empty vesicles plus a population of vesicles containing a small number of shift reagent molecules. This seems unlikely intuitively and because the NMR signal had collapsed after a waiting period in the absence of shift reagent (and shift reagent only then added for monitoring purposes).

JA065702O