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Squalane is in the midplane of the lipid bilayer: implications for its function as a proton permeability barrier

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Abstract

A recently proposed model for proton leakage across biological membranes [Prog. Lipid Res. 40 (2001) 299] suggested that hydrocarbons specifically in the center of the lipid bilayer inhibit proton leaks. Since cellular membranes maintain a proton electrochemical gradient as a principal energy transducer, proton leakage unproductively consumes cellular energy. Hydrocarbons in the bilayer are widespread in membranes that sustain such gradients. The alkaliphiles are unique in that they contain up to 40 mol% isoprenes in their membranes including 10–11 mol% squalene [J. Bacteriol. 168 (1986) 334]. Squalene is a polyisoprene hydrocarbon without polar groups. Localizing hydrocarbons in lipid bilayers has not been trivial. A myriad of physical methods including fluorescence spectroscopy, electron-spin resonance, nuclear magnetic resonance as well as X-ray and neutron diffraction have been used to explore this question with various degrees of success and often contradictory results. Seeking unambiguous evidence for the localization of squalene in membranes or lipid bilayers, we employed neutron diffraction. We incorporated 10 mol% perdeuterated or protonated squalane, an isosteric analogue of squalene, into stacked bilayers of dioleoyl phosphatidyl choline (DOPC) doped with dioleoyl phosphatidyl glycerol (DOPG) to simulate the negative charges found on natural membranes. The neutron diffraction data clearly show that the squalane lies predominantly in the bilayer center, parallel to the plane of the membrane.

Keywords: Proton leak; Squalene; Squalane; Proton gradient; Ubiquinone; Neutron diffraction; Lipid bilayer

1. Introduction

Polyisoprenes are components of natural membranes that are poorly understood. Neither the function of polyisoprenes as a class of natural membrane lipids nor their location in the lipid bilayer has been established experimentally. Many polyisoprenes contain quinones, which are used for oxidation–reduction reactions and as cofactors for the movement of protons by proteins. Although the role of the quinone group is well established, the function and location of the polyisoprene domain of these molecules in membranes remains a mystery. All biological membranes that maintain proton gradients contain polyisoprenes except for those membranes that contain phytosterols, plant sterols that differ from cholesterol in that they have branches in the center of the bilayer. The best known of these polyisoprenes is ubiquinone in the mitochondrion and in prokaryotes [1] and plastoquinone and the myriad of isoprenes associated with the chloroplast and prokaryote photosynthetic membranes.

A prediction of the "water cluster" model [2] for proton leakage across lipid bilayers suggests that any hydrocarbon in the center of lipid bilayers may serve as an inhibitor of proton leakage in addition to any other functions it may serve for the cell. This may include either branching of the lipids at or near the bilayer center or hydrocarbons resident in the bilayer center. Prior modeling of proton leakage across bilayers has been a "water wire" [3–5] in which some 8 to 15 water molecules form a hydrogen-bonded string across the bilayer to conduct protons across the low

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dielectric. The "water cluster" model [2] for proton leakage is based on protonated and deprotonated clusters of water in the low dielectric of each monolayer that must contact each other across the center of the bilayer in order for protons to be transferred across the bilayer. This latter proposal implies that hydrocarbons such as polyisoprenes that may reside in the center of the bilayer inhibit proton leakage. To test this hypothesis, it is necessary first to establish the location of prospective hydrocarbons within lipid bilayers. Neutron diffraction, in combination with specifically deuterated samples, is ideal for such experiments.

Squalene (Fig. 1), a polyisoprene that is best known as a critical precursor for cholesterol, is present in the membranes of alkaliphiles [6] which are especially sensitive to proton leaks. These prokaryotes have a constant cytosolic pH (8.3) and ATP synthesis requires pumped protons from the alkaline environment [7]. The mole fraction of squalene in alkaliphile membranes is around 10–11% whereas it and other isoprenes are absent from the control neutrophile, *Bacillus subtilis*. Furthermore, the alkaliphile membranes contain some 40 mol% polyisoprenes including tetrahydrosqualene, squalene, and unidentified C40 and C50 polyisoprenes. All are absent from the neutrophile. These data suggest that the function of squalene and the other polyisoprenes in these organisms may be to inhibit proton leakage across the plasma membrane. It is therefore of value to determine if the squalene lies in the bilayer center. Squalane (Fig. 1) is especially appropriate for localizing in membranes the polyisoprene domains of lipids such as ubiquinone, plastoquinone, dolichol and others, since it does not contain polar groups and has the same branched chain structure. Using neutron diffraction, we have demonstrated that squalane, an analogue of squalene (see Fig. 1), lies in the center of stacked, synthetic lipid bilayers of dioleoyl phosphatidyl choline (DOPC) doped with dioleoyl phosphatidyl glycerol (DOPG), to simulate the negative charges found on natural membranes.

2. Experimental procedures

2.1. Materials

Perdeuterated squalane (2,6,10,15,19,23-hexamethyltetracosane-d_{62}), 98% purity was purchased from CDN Isotopes, (Pointe-Claire, Canada). Protonated squalane was obtained from Aldrich. DOPC and the net negatively charged lipid DOPG were products of Avanti Polar Lipids, Alabaster, AL.

2.2. Preparation of stacked bilayers

Two chloroform solutions containing DOPC/DOPG, 82:9 mol%, and an additional 9 mol% squalane, the latter either protonated or deuterated, were prepared. The total lipid weight for each preparation was 20 mg. Oriented samples were obtained by spraying the solutions, using an artist's airbrush (aerobrush pro281 obtained from Hansa Norderstedt, Germany), on quartz slides ($65 \times 15 \times 0.3$ mm), covering only the central area of one side. The reservoir of the airbrush was filled with 1 ml of the chloroform solution containing the lipid mixture and slowly sprayed with a low pressure of 1.5 bar, obtaining homogenous thin lipid films. Residual traces of solvent were removed by placing the slides in a vacuum desiccator for 12 h ($p < 1$ mbar). Samples were then rehydrated for 24 h at room temperature in an atmosphere of 98% relative humidity, maintained with a saturated K_2SO_4 solution.

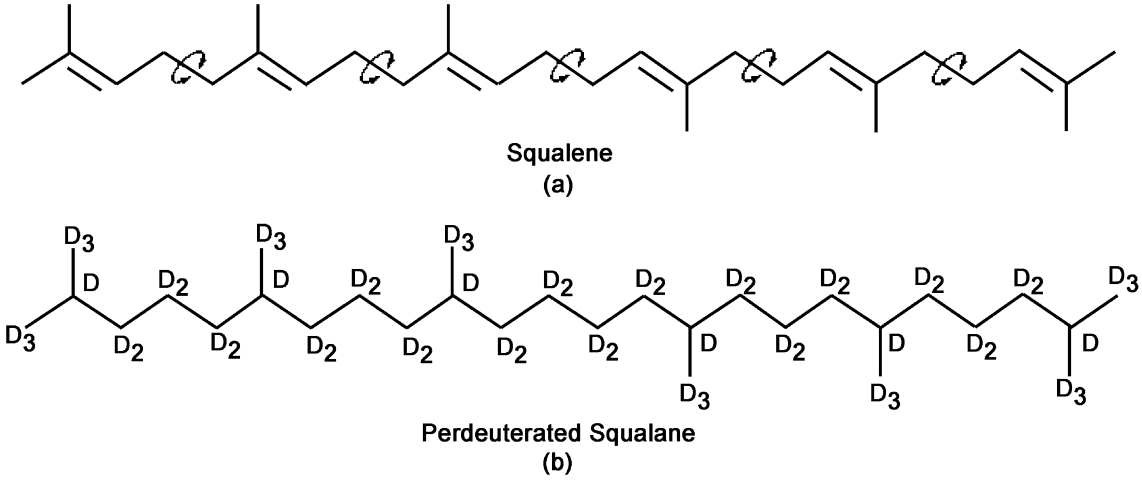

Fig. 1. Structures of squalene (a) and perdeuterated squalane (b). Squalene, with its isolated double bonds consists of a series of hinged planes. A double arrow indicates the bond hinges. The planes include all of the methyl branches. The planes place constraints on the distributions of its orientations in the lipid phase. In contrast, squalane, consisting entirely of sp_3 carbons, has no such constraints. Squalene's constraints implies that it is even more likely than squalane to remain in the bilayer center because squalene will disturb the cooperative motions of the phospholipid chains to a greater extent than squalane.

2.3. Neutron diffraction

Neutron diffraction measurements were carried out on the membrane diffractometer V1 at the Berlin Neutron Scattering Center of the Hahn-Meitner-Institute, Berlin (Germany). Samples containing either protonated or deuterated squalane were placed in thermostated aluminum cans, in which the humidity was controlled by aqueous saturated K_2SO_4 solutions in Teflon reservoirs. Contrast variation was achieved by adjusting the atmosphere in the sample can to three different D_2O/H_2O molar compositions (50:50, 20:80 and 8:92). After each change of the aqueous solution, the samples were allowed to equilibrate for 24 h.

Diffraction intensities were measured at $T=27$ °C with rocking scans, rocking the sample around the expected Bragg position θ by $\theta \pm 2°$. Up to six orders were detected for each sample. Diffraction patterns of DOPC/DOPG bilayers containing 10 mol% deuterated and protonated squalane, respectively, were measured (Fig. 1). The lamellar spacing d of each sample was calculated by least-square fitting of the observed 2θ values to the Bragg equation $n\lambda = 2d\sin\theta$, where n is the diffraction order and λ is the neutron wavelength (4.52 Å).

Integrated intensities were calculated with a Gaussian fit of the experimental Bragg reflections. The square-roots of the intensities, corrected with absorption and Lorentz factors, produced the structure factor amplitudes. The measured intensities were scaled with the mass of the dried samples. The phase assignment was obtained with the isomorphous replacement method, using the D_2O/H_2O exchange and assuming a Gaussian distribution of the water layer [8].

The scattering length density profile $\rho(z)$ is given by:

$$\rho(z) = \rho_0(z) + \frac{2}{d}\sum_{h=1}^{n} f(h)\cos\frac{2\pi hz}{d}$$

where ρ_0 (z) is the average density per unity length of the bilayer, $f(h)$ are the scaled structure factors and the second term describes the distribution in scattering lengths across the bilayer. For more details to evaluate the localization of labels in biological membranes, see Ref. [9].

3. Results

3.1. Neutron diffraction

The rocking scans around the first-order peaks display a mosaicity inferior to 0.5° demonstrating the excellent quality of the samples. Fig. 2 shows the diffraction patterns obtained from the two samples containing perdeuterated (solid line) and protonated (dotted line) squalane at different H_2O/D_2O contrasts. Up to six diffraction orders for each sample can be seen. The calculated d-spacing for the two samples was the same within experimental error. It was

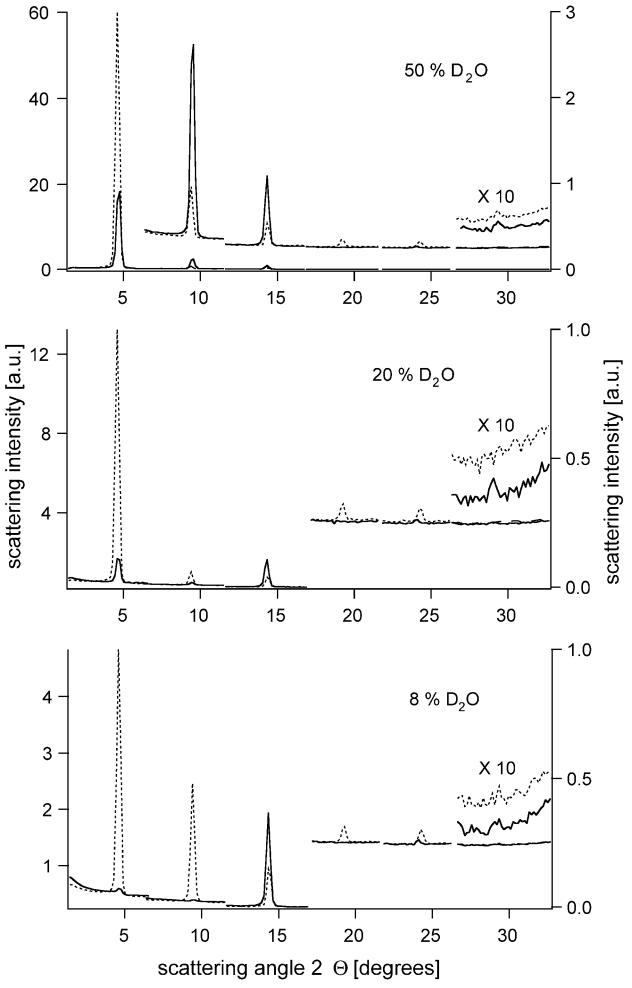

Fig. 2. Diffractograms of the DOPC/DOPG (90:10 mol%) sample containing protonated squalane (dotted line) and deuterated squalane (solid line), respectively, at different D_2O/H_2O contrasts. Up to six orders are visible. Pronounced differences of the peak's relative intensities can be seen for the two samples. In particular, at 8% D_2O content, the diffractogram of the sample containing deuterated squalane is dominated by the third order peak. The sixth order is zoomed by a factor of 10 to the right intensity scale and an arbitrary offset was added.

equal to 53.1 ± 0.4 Å. The fact that the d spacing and the distance between the glycerol backbones do not vary between the samples of protonated and deuterated squalane supports the approach of directly comparing the samples. Pronounced relative intensity changes can be observed as the two samples are compared (Fig. 2). This contrast could be observed (equivocal antecedent) from examining the raw data without calculating the difference spectrum. Inspection of the diffraction patterns at 8% D_2O atmosphere suggests that the deuterated squalane is located in the center of the membrane. The third-order reflection dominates the diffraction pattern for the sample with deuterated squalane. This results in a scattering density maximum in the center of the unit cell. Note that deuterium is a much stronger coherent scatterer (6.7 fm) as compared to hydrogen (−3.7 fm). Profiles of the scattering length densities (i.e., the membrane

profiles) are calculated as the Fourier sum of the structure factors. They are reported for 8% D_2O in Fig. 3. The unit cell is chosen so that the midplane of the lipid membrane is in the center and the water layers are at its outer edges.

The 8% D_2O contrast was chosen for representation in Fig. 3 because at this H_2O/D_2O ratio, the mean scattering length of the water is zero. A typical lipid membrane profile at this contrast shows two maxima that are dominated by the glycerol backbone of the lipids (and therefore represent the lipid headgroups) and a minimum at the bilayer center, corresponding to the terminal methyl groups. The sample containing protonated squalane resembles this profile, whereas the sample with deuterated squalane shows a profile with a pronounced maximum in the middle of the unit cell. Therefore, even without calculating the difference between the two samples, the location of squalane is unambiguously localized to the bilayer center due to the strong scattering of the 62 deuterons used as an isomorphic label in the squalane. The difference between the profiles of the samples with deuterated and protonated squalane gives the location of the deuterated squalane label. In Fig. 3B, this difference is clearly seen in the middle of the lipid bilayer. The lateral peaks in Fig. 3B are attributed to the truncation error of the Fourier sum.

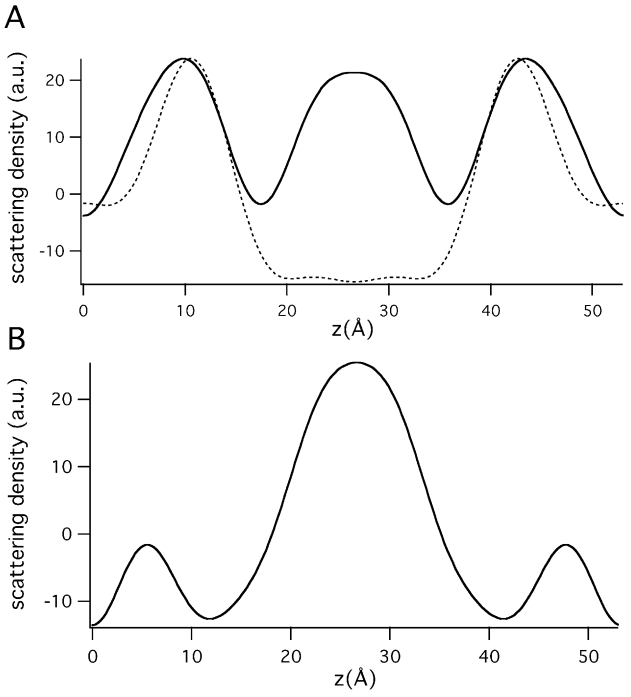

Fig. 3. (A) Scattering density profiles of the DOPC/DOPG (90:10 mol%) membrane containing protonated (dotted line) or deuterated squalane (solid line) 8% D_2O contrast. (B) Deuterium distribution calculated as difference between the scattering density profiles of the membrane containing deuterated or protonated squalane. The central maximum gives the position of the deuterated squalane, in the middle of the unit cell, i.e. in the center of the membrane. The two lateral peaks are due to the truncation error of the Fourier sum.

4. Discussion

4.1. Neutron diffraction

In order to compare the scattering density profiles of the samples with protonated and deuterated squalane, the two curves in Fig. 3A have been superimposed so that they coincide in the headgroup region, where it was obvious that the deuterated label was not located. From inspection of Fig. 3A, an apparent broadening of the head group region for the sample containing deuterated squalane is visible. For this sample, however, the Fourier sum is heavily dominated by the third order term; orders beyond the sixth, which can result in a better modulation of the membrane profile, were not measured due to limited beamtime.

Information about the membrane thickness can be derived from the positions of the density peaks corresponding to the lipid headgroups. In our case, i.e. DOPC/DOPG membrane containing squalane, the distance between headgroups was 33.8 Å. A similar mixture of DOPC/DOPG was investigated by Bradshaw [10], although at 100% relative humidity, instead of 98% as in our case. Using Fig. 2 of the cited paper, we measured the distance between the headgroup regions (33.5 Å). Compared to our value of 33.8 Å, we infer that the squalane does not increase the membrane thickness. On the other hand, the repeat distances of the respective bilayers were quite different (53.1 Å in our case, 48.4 Å in Ref. [10]) probably due to the difference in the water content in the sample.

Due to the high quality of the neutron diffraction data and the very strong label used for our experiments, both from the scattering density profile of the deuterated sample (Fig. 3A) and from the difference density profile (Fig. 3B), the location of squalane in the bilayer center is demonstrated unambiguously.

4.2. Localization of squalane in bilayers

Studies on the orientation of hydrocarbon molecules in bilayers date back to the beginning of our understanding of bilayer structure. In the studies by Haydon et al. [11,12] in the 1970s, the orientation of various *n*-hydrocarbons was established in defined lipid bilayers. They used two principles. They measured changes in the thickness of the bilayer, which they interpreted as demonstrating that the hydrocarbon was in the center plane of the bilayer; and they measured surface tension changes, which they interpreted as the hydrocarbon standing erect, i.e., parallel to the membrane lipid chains. By this combination of studies, they concluded that *n*-octane and shorter chains reside in the center of the bilayer, whereas *n*-alkanes between 8 and 20 carbons are erect, i.e., parallel to the chains of the phospholipids. *N*-alkanes longer than 20 carbons again reside in the bilayer center. This contrasts with straight-chain alcohols [13], which, regardless of chain length, are oriented so that the hydroxyls are in the headgroup domain and the chains are parallel to the chains of the lipids.

Our data in Fig. 3 prove that squalane is oriented parallel to the plane of the bilayer and at its center, i.e., sandwiched between the two monolayers (Fig. 4). The relatively broad label distribution might arise from the flexibility of the squalane molecule, which may partially extend into the lipid acyl chains. All biological membranes, except for the diphytanyl chains of the archaebacteria, have straight-chain lipids.

Indirect measurements of the localization of polyisoprenes in bilayers have been based on measurements of the T_m of bilayers using differential scanning calorimetry. The T_m is determined in a bilayer by each monolayer independently because of the cooperative lateral motions of the chains in each monolayer. Thus, an unchanged T_m suggests that the additive is in the cleavage plane and not in either monolayer. For example, Katsikakis and Quinn [14,15] concluded that using this approach, coenzyme Q_{10} at up to 20 mol% codispersions in dipalmitoyl phosphatidyl choline (DPPC) lies in the center of the bilayers. Using fluorescence quenching, they drew the same conclusion [16]. Dolichol (phosphate-free), which is found in lysosomes, Golgi and peroxisomes [17], also does not affect the T_m of phospholipid bilayers that contain it [18]. Finally, Quinn [19] has shown, using X-ray diffraction, that tocopherol, another polyisoprene, thickens the bilayer and concluded that it is also in or near the bilayer center.

Our data were obtained using perdeuterated squalane. As illustrated in Fig. 1, squalane differs from squalene in that the squalane domains have considerably fewer constraints on the distributions of the orientations in the lipid phase due to the absence of double bonds. Squalene, like all polyisoprenes, consists of a series of hinged planes. All the carbons in squalene are in one of these planes. We have found the squalane, with all of its carbons in the flexible sp_3 configuration, to be predominantly in the bilayer center. The more constrained squalene, with its hinged planes, would interfere more than squalane with the cooperative motions of the straight-chain lipids that form the bilayer. Squalene is more likely to remain in the bilayer center than squalane.

Two features of straight-chain lipid bilayers seem to provoke the polyisoprenes to reside in the bilayer center. First, the cooperative motions of the chains in each monolayer are disrupted by intrusion of branched hydrocarbons with each monolayer, more so when the branched hydrocarbon consists of a series of hinged planes. Second, the two monolayers are uncoupled so that the lateral motions of the chains within one monolayer do not affect the motions of the other.

Using neutron diffraction, it was possible for the first time to provide direct evidence that the polyisoprane, squalane, is predominantly in the center of the bilayer (Fig. 4).

4.3. Implications for isoprenes in biological membranes

Except for animal plasma membranes, which maintain a sodium gradient, all natural membranes maintain proton gradients. All of these membranes have a $\Delta\Psi$ across the membrane so oriented that it is expected to increase the proton leakage. Leakage is wasted energy as it requires ATP to restore the $\Delta\Psi$ [20]. Paula et al., [21] have demonstrated, using bilayers made of phosphatidyl choline lipids with chains varying from C_{12} to C_{22}, that the proton leakage is exponentially inversely proportional to the thickness of the bilayer.

All eukaryote membranes that maintain proton gradients contain polyisoprenes except for those plasma membranes that contain phytosterols, which have branches in the center of the bilayer. Examples of the distribution of these nonbilayer-forming lipids include ubiquinone in the mitochondrion, plastoquinone in the chloroplast and, perhaps, surprisingly, free dolichol in the lysosome, Golgi, endoplasmic reticulum and peroxisome [17,22–24].

Proton gradients together with ATP are the interconvertible storage forms of energy in living cells. Plants, eukaryote microbes, such as yeast and fungi, and eukaryote organelles use proton gradients to power transport processes including nutrition, excretion, ATP synthesis and many other functions. Cation (proton or sodium) gradients and ATP are two energy resources that together supply the total energy of living cells. There is a balance between them in all cells. Thus, any leakage of these cations decreases the amount of the cell's ATP.

Direct measurement of the rate at which protons leak across lipid bilayers at biologically important fluxes (10^{-5} cm/s) was made in 1980 [5,25]. This has been confirmed by many laboratories [26,27] and has recently been reviewed [28]. These references include numerous citations of the

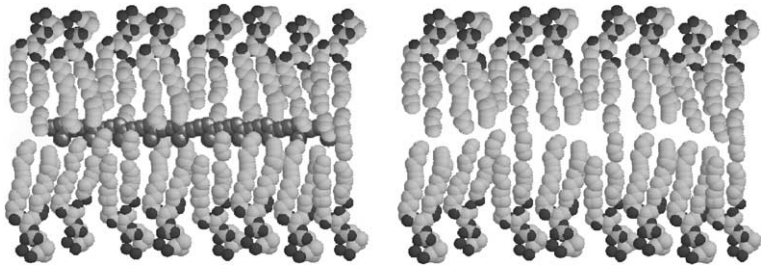

Fig. 4. Schematic representation of squalane in the bilayer center as demonstrated by the neutron diffraction data (left) and a representation of the bilayer without squalane (right).

significance of proton leaks to understanding the functioning of mitochondria.

Prokaryote alkaliphiles synthesize squalene and squalene dimers and trimers [6]. Surprisingly, the squalene synthesis is increased as the pH of the culture medium is increased. At high pH, isoprenes, including squalene, constitute over 40 mol% of the lipids. Our data show that squalene and the polyisoprene domain of natural polyisoprenes lie in the center of the bilayer. The observations of a pH-induced increase of squalene (and other polyisoprenes) in the membranes of the alkaliphiles taken together with our localization of squalane in the bilayer suggests that polyisoprenes may inhibit proton leakage.

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