Individual Leaflets of a Membrane Bilayer Can Independently Regulate Permeability*

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Water rapidly crosses most membranes, but only slowly across apical membranes of barrier epithelia such as bladder and kidney collecting duct, a feature essential to barrier function. How apical membrane structure reduces permeabilities remains unclear. Cell plasma membranes contain two leaflets of distinct lipid composition; the role of this bilayer asymmetry in membrane permeability is unclear. To determine how asymmetry of leaflet composition affects membrane permeability, effects on bilayer permeation of reducing single leaflet permeability were determined using two approaches: formation of asymmetric bilayers in an Ussing chamber, with only one of two leaflets containing cholesterol sulfate, and stabilization of the external leaflet of unilamellar vesicles with praseodymium (Pr³⁺). In both systems, permeability measurements showed that each leaflet acts as an independent resistor to water permeation. These results show that a single bilayer leaflet can act as the barrier to permeation and provide direct evidence that segregation of lipids to create a low permeability extracellular leaflet may act to reduce the permeability of barrier epithelial apical membranes.

Epithelial cells generate and maintain apical membrane bilayers made up of leaflets of distinct composition by mechanisms involving asymmetric biosynthesis in the Golgi, oriented insertion into the plasma membrane, and the activity of ATP-driven phospholipid flippases (1–6). In several epithelia, such as bladder and kidney collecting duct, there is a structural feature such as bladder and kidney collecting duct, a feature essential to barrier function. How apical membrane structure reduces permeabilities remains unclear. Cell plasma membranes contain two leaflets of distinct lipid composition; the role of this bilayer asymmetry in membrane permeability is unclear. To determine how asymmetry of leaflet composition affects membrane permeability, effects on bilayer permeation of reducing single leaflet permeability were determined using two approaches: formation of asymmetric bilayers in an Ussing chamber, with only one of two leaflets containing cholesterol sulfate, and stabilization of the external leaflet of unilamellar vesicles with praseodymium (Pr³⁺). In both systems, permeability measurements showed that each leaflet acts as an independent resistor to water permeation. These results show that a single bilayer leaflet can act as the barrier to permeation and provide direct evidence that segregation of lipids to create a low permeability extracellular leaflet may act to reduce the permeability of barrier epithelial apical membranes.

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TABLE I

Effect of varying leaflet composition on water permeability

| Water permeabilities of bilayers | (n = 3) |
|---------------------------------|--------|
| cm²/s × 10⁻⁴                     | S.D.   |
| DPhPC/DPhPC                     | 14.9 ± 1.7 |
| DPhPC-CS/DPhPC-CS               | 5.4 ± 0.5  |
| DPhPC/DPhPC-CS                 | 8.9 ± 1.6  |
| Calculated value of DPhPC single leaflet permeability | 29.8 |
| Calculated value of DPhPC-CS single leaflet permeability | 10.8 |
| Expected value of DPhPC/DPhPC-CS bilayer permeability | 7.9 |

Effect of varying leaflet composition on water permeability

On measurements of permeability of bilayers, DPhPC and DPhPC-CS bilayers were used. It was found that the measured permeability of DPhPC bilayers was 1.6 cm²/s, a value indistinguishable from the expected value obtained with Pr₃⁻. The presence of CS in both leaflets markedly reduced the permeability of the symmetric bilayer system. In the presence of CS, the permeability of the symmetric bilayer system was reduced to 0.03 cm²/s, a value indistinguishable from the expected value obtained with Pr₃⁻. The presence of CS in both leaflets markedly reduced the permeability of the symmetric bilayer system. In the presence of CS, the permeability of the symmetric bilayer system was reduced to 0.03 cm²/s, a value indistinguishable from the expected value obtained with Pr₃⁻.

RESULTS AND DISCUSSION

To determine the effects of single leaflet structure on bilayer permeability, P_d was measured using tritiated water and values corrected for unstirred layer effects are shown in Table I. To correct for unstirred layer effects, [¹⁴C]butanol fluxes were performed in a manner identical with water fluxes. Because butanol is highly permeable across membranes, its flux measures the thickness of the unstirred layer. Pr3⁻ was added to the outside of liposomes to reduce the fluidity of the external leaflet. Pr₃⁻ complexes with the phosphate head groups of phospholipid molecules, reducing their mobility and that of the adjacent hydrocarbon chains. This stabilization has been detected previously as an increase in the phase transition temperature of the outer leaflet of vesicles using NMR measurements. To examine the fluidity of the outer leaflet, we introduced a phospholipid-bound probe of anisotropy, 2-(3-(diphenylhexatrienyl)propanyl)-1-hexadecanoyl-sn-glycerol-3-phosphocholine (DHP HPC) into the outer leaflet, and monitored fluorescence anisotropy as a function of temperature. Because phospholipids added to the outside of the liposomes enter the outer leaflet rapidly but cross to the inner leaflet slowly, this probe monitored exclusively the outer leaflet of the bilayer. As shown in Fig. 1A, addition of Pr₃⁻ raised the transition temperature for this probe by 2–3 °C, in good agreement with the magnetic resonance studies. Fig. 1, B and C, shows the effect of Pr₃⁻ on water permeability at varying temperatures. In the absence of Pr₃⁻, P_d rose abruptly at 42 °C, and leveled off at values of 0.03 cm²/s, corresponding to the known phase transition temperature of DPPC. Following addition of Pr₃⁻ to the same LUV, P_d was similar to control values at temperatures well below and above the phase transition temperature. However, the phase transition temperature for Pr₃⁻ was raised by 2–3 °C, so that Pr₃⁻ markedly reduced P_d at temperatures in the vicinity of the transition temperature for control LUV. These results indicate that reducing the fluidity of a single leaflet reduces the permeability of water across the entire bilayer.

To determine whether the resistance equation applies as well to the LUV exposed to Pr₃⁻, leaflet A (DPPC without Pr₃⁻) permeabilities were calculated from the values for control LUV at temperatures above the phase transition. Leaflet B (DPPC + Pr₃⁻) permeabilities (P_B) were estimated by taking the anisotropy value obtained with Pr₃⁻ and applying that value to a standard curve relating permeability to anisotropy in control LUV. Because we have previously shown that DPH anisotropy is directly related to measured water permeability (11), this approach should give an accurate estimate of the water permeability of leaflet B. Using these P_A and P_B values, we calculated expected values for P_BL, and these expected values are shown in Fig. 1C (as filled squares). It is apparent that this equation applies equally well to diffusive water permeability in bilayers made asymmetric with CS and to osmotic water permeability in bilayers made asymmetric with CS and to osmotic water permeability in
Bilayer Asymmetry and Membrane Permeability

We have previously reconstituted lipids quantitatively extracted from gastric apical membrane vesicles and shown that the reconstituted lipids newly arranged in artificial liposomes do not reconstitute low water permeability (15). The failure of the extracted lipids when reconstituted into artificial liposomes to duplicate the low permeabilities of the intact membrane may be due to the influence on permeability of two factors in the intact membrane: the presence of integral membrane proteins or the arrangement of the lipid components of the membrane into asymmetrical leaflets (15). Our new results show directly that segregation of lipid of low fluidity in a single leaflet of the bilayer reduces the permeability of the entire bilayer. Therefore, we can anticipate that cells create apical membranes of low permeability by segregating phospholipid molecules with long saturated hydrocarbon chains and cholesterol in the outer leaflet of the bilayer. Indeed, where the composition of individual bilayer leaflets has been examined, this segregation has been observed (26–28). Such segregation of lipids is important in determining the low permeability properties of the apical as opposed to the basolateral membrane domain, because, in epithelial cells, lipids of the exofacial but not cytoplasmic leaflet of the apical membrane are prevented from mixing with those of the corresponding basolateral membrane leaflets by the tight junctions (3, 6). These considerations plus the evidence from the current study indicate that the lower permeabilities of apical as compared with basolateral membrane domains are due to the lipid structure of the exofacial leaflet.

Cells invest a great deal of effort to generate and maintain blayers with distinct leaflet compositions. Membrane biosynthesis in the Golgi apparatus occurs in an asymmetric fashion, with distinct lipids going into the different membrane leaflets (1–3). Moreover, cells maintain phospholipid “flippases,” in their plasma membranes (4, 5). These transporters couple ATP degradation to the movement of phospholipid molecules from one leaflet to the other, a process which is otherwise energetically unfavorable and occurs at a very slow rate. Our studies provide direct biophysical evidence that the generation and maintenance of asymmetric apical membranes can result in effective barrier function.

The present studies emphasize the importance of segregation of low fluidity lipids in the exofacial leaflet for maintenance of low permeability. Any disease process which disrupts the ability of cells to create and maintain membranes with distinct cytoplasmic and exofacial leaflets may result in failure of apical membrane barrier function, with resulting damage to subepithelial structures or loss of homeostatic function. Candidate diseases which may disrupt the generation and maintenance of asymmetric bilayer structure include ulcer disease in the stomach, cystitis in the bladder, and renal tubular acidosis and inability to concentrate or dilute the urine in the kidney collecting duct.

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Fig. 1. Effect of external Pr3+ on phase transition temperature for outer leaflet fluidity and for osmotic water permeability (Pf) of DPPC liposomes. A, fluidity of the outer leaflet was monitored using DPH HPC (excitation 360 nm, emission 430 nm) at a probe/lipid ratio of 1:400 at varying temperatures in the absence (filled circles) and presence (filled squares) of 10 mM extravesicular Pr3+ on a SPEX spectrofluorimeter using standard methods (24, 25). B, measurements of water flux at 44°C in the absence (DPPC) and presence (DPPC/Pr3+) of 10 mM extravesicular Pr3+. Data from 6–10 curves were averaged and fitted as described; averaged data and fitted curves are shown. C, effect of extravesicular Pr3+ on Pf at varying temperatures. Pf was calculated from curves similar to those of B at varying temperatures as described in the absence and presence of 10 mM Pr3+. Data from 6 different experiments are shown. Values obtained with Pr3+ differ significantly from those obtained in the absence of Pr3+ at 42–46°C, by t test.

Previous studies had provided evidence that individual leaflets of membrane bilayers could alter their physical properties independent of the other leaflet (23, 24). Our results demonstrate for the first time that individual leaflets of the membrane bilayer can independently regulate permeation. If the permeabilities of both leaflets are similar, then both will contribute similarly to membrane permeability. By contrast, if one of the leaflets has a very low permeability, the permeability of this leaflet will predominate, so that the permeability of the entire bilayer will be close to that of the low permeability leaflet. This conclusion has several important implications for our current understanding of the role of bilayer asymmetry in epithelial cell biology.

liposomes made asymmetric with Pr3+.
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