Water and solute permeability of rat lung caveolae: high permeabilities explained by acyl chain unsaturation


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Hill, Warren G., Eyad Almasri, W. Giovanni Ruiz, Gerard Apodaca, and Mark L. Zeidel. Water and solute permeability of rat lung caveolae: high permeabilities explained by acyl chain unsaturation. *Am J Physiol Cell Physiol* 289: C33–C41, 2005. First published February 23, 2005; doi:10.1152/ajpcell.00046.2005.—Caveolae are invaginated membrane structures with high levels of cholesterol, sphingomyelin, and caveolin protein that are predicted to exist as liquid-ordered domains with low water permeability. We isolated a caveola-enriched membrane fraction without detergents from rat lung and characterized its permeability properties to nonelectrolytes and protons. Membrane permeability to water was 2.85 ± 0.41 × 10⁻³ cm/s, a value 5–10 times higher than expected based on comparisons with other cholesterol and sphingolipid-enriched membranes. Permeabilities to urea, ammonia, and protons were measured and found to be moderately high for urea and ammonia at 8.85 ± 2.40 × 10⁻⁴ cm/s and 6.84 ± 1.03 × 10⁻⁴ cm/s respectively and high for protons at 8.84 ± 3.06 × 10⁻³ cm/s. To examine whether caveolin or other integral membrane proteins were responsible for high permeabilities, liposomes designed to mimic the lipids of the inner and outer leaflets of the caveolar membrane were made. Osmotic water permeability to both lipidic compositions were determined and a combined inner/outer leaflet water permeability was calculated and found to be close to that of native caveolae at 1.58 ± 1.1 × 10⁻³ cm/s. In caveolae, activation energy for water flow was high (19.4 kcal/mol) and water permeability was not inhibited by HgCl₂; however, aquaporin 1 was detectable by immunoblotting. Immunostaining of rat lung with AQP1 and caveolin antisera revealed very low levels of colocalization. We conclude that aquaporin water channels do not contribute significantly to the observed water flux and that caveolae exhibit low water and solute permeabilities. First, as a naturally occurring membrane rich in lipid raft components, would caveolae exhibit low water and solute permeabilities? Second, what, if any, is the physical impact of the caveolin protein “cage” embedded in and surrounding this membrane? Third, what, is the physical impact of the caveolin protein “cage” embedded in and surrounding this membrane? Third, could we confirm the presence of functional water channels in this organelle (34)? Our results showed that caveola-enriched membranes (CEM) are surprisingly permeable to water and solutes and appear to have a relatively high permeability to protons. We could show no evidence for aquaporin-mediated water transport despite the presence of low levels of aquaporin 1 (AQP1) as established by immunoblotting.

The contribution of caveolin protein was investigated indirectly by making liposomes which mimic the lipid composition of caveolae. Water permeability in artificial membranes was close to that of native caveolae, suggesting that membrane-embedded caveolin and other integral membrane proteins do not dramatically perturb lipid packing, or alternatively, rigidify the membrane. These experiments highlight several unexpected properties of caveolae and raise important questions regarding the precise distribution of cholesterol, its interaction with caveolin, and the functional relevance of AQP1 in this microdomain.

**MATERIALS AND METHODS**

**Buffers.** Three buffers were used, composed of the following: 0.25 M sucrose, 1 mM EDTA, 20 mM Tricine, pH 7.8 (buffer A); 0.25 M...
sucre, 6 mM EDTA, 120 mM Tricine, pH 7.8 (buffer B); and 50% Optiprep (Accurate Chemical and Scientific, Westbury, NY) in buffer B (buffer C).

Isolation of caveolae from rat lung. Caveolae were isolated from rat lung essentially using the method described by Smart et al. (38), except that the starting material was tissue rather than cultured cells. Lungs from two adult Sprague-Dawley female rats were removed from anesthetized animals, minced finely with a razor blade, and then homogenized by 20 strokes in a motorized Potter-Elvehjem tissue homogenizer in 8 ml of ice-cold buffer A containing protease inhibitors (Complete Mini, Roche) and 10 mM 5,6-carboxyfluorescein (Molecular Probes, Eugene, OR). Carboxyfluorescein acts as an entrapped fluorescent volume and pH reporter. All subsequent steps were carried out on ice or at 4°C. The homogenate was centrifuged at 1,000 g for 5 min and then was divided in two and layered on top of two tubes containing 30% Percoll (Amersham Biosciences, Piscataway, NJ) in buffer A (23 ml) and centrifuged at 84,000 g for 35 min. The plasma membrane fraction seen at the interface between red (due to hemolysis) and clear solutions was recovered with the use of a gradient maker and peristaltic pump and then overlayed with 2.8 ml 5% Optiprep (prepared by diluting appropriate volumes of buffer A with buffer C, total gradient volume of 12 ml) was then poured on top of a gradient (prepared by diluting appropriate volumes of buffer A in buffer C, total gradient volume of 12 ml) and then poured on top of the membranes using a gradient maker and peristaltic pump and centrifuged at 52,000 g for 95 min in a swinging bucket rotor. The top 8 ml of the gradient (fractions 1–5 of 15) were diluted with 6.4 ml buffer C, then overlayed with 2.8 ml 5% Optiprep (prepared by diluting buffer C with buffer A), and centrifuged at 52,000 g for 95 min. An opaque band of CEM was present at the interface between the two Optiprep concentrations on completion of the run. The fractions (12 × 1.4 ml) were collected and aliquots analyzed by immunoblotting for caveolin-1. The majority of caveolin-1 was usually found in fraction 3. CEM to be used for stopped-flow fluorometry were pelleted by centrifugation at 100,000 g for 1 h and then resuspended in buffer A.

Fluorometry. Before stopped-flow measurements vesicles were tested for osmotic responsiveness with a luminescence spectrometer (Amino Bowman Series 2). The addition of defined hyper- or hypoosmotic solutions to diluted vesicles resulted in appropriate reductions or increases in the fluorescence of vesicles in response to volume alteration. Fluorometry was performed at excitation/emission wavelengths of 490/520 nm.

Liposome formation. Purified lipids in chloroform (Avanti Polar Lipids, Alabaster, AL) were mixed in the appropriate molar ratios, dried under nitrogen for 20 min, and then vacuum dried for 2 h. Dried lipids were resuspended in 150 mM NaCl, 10 mM HEPES, 1 mM DTT, and 5 mM carboxyfluorescein, pH 7.4, and vortexed for 2 min. Outer leaflet lipids were probe sonicated in 30-s bursts six times with 2-min intervals on ice in between. Sonication was followed by extrusion through a 200-nm polycarbonate filter. Inner leaflet lipids with less cholesterol were bath sonicated three times for 2 min at a time with 2-min intervals on ice. Extravesicular carboxyfluorescein was removed by Sephadex G25 gel filtration and then liposomes were incubated on ice for 2 h. Outer leaflet liposomes were 27 ± 67 nm in diameter while inner leaflet liposomes were 144 ± 15 nm in diameter (n = 3 preparations). Liposomes were always tested on the same day they were made.

Water, urea, ammonia, and proton permeability measurements. Permeabilities to water, urea, ammonia, and protons were measured at 25°C by stopped-flow fluorometry as previously described (6, 17, 32). Briefly, permeabilities were determined with the use of a stopped-flow fluorometer (model SX.18 MV, Applied Photophysics, Leatherhead, UK) with a measurement dead-time of <1 ms. To determine osmotic water permeability (P), caveolae or liposomes containing carboxyfluorescein (10 and 5 mM, respectively) were rapidly mixed with hypertonc buffer A (adjusted to three times the original osmolality with sucrose). Buffer osmolalities were determined with the use of an osmometer (Osmette A; Precision Systems, Natick, MA). The rate of water efflux from vesicles was measured as a function of vesicle shrinkage and carboxyfluorescein self quenching. Typically, 8–10 quench curves were averaged and fit to a single exponential curve. To ensure that the only signal recorded was from intravesicular fluorescence, fluorescein-quenching antibody (Molecular Probes) was added before the experiment. From parameters which included the initial rate of quenching, vesicle diameter and applied osmotic gradient, P was calculated using MathCad software (MathSoft, Cambridge, MA). In some experiments, 10 mM methyl-β-cyclodextrin (Sigma, St, Louis, MO) was added for 30 min at room temperature before stopped-flow experiments. Urea permeabilities were assayed by incubating CEM in buffer A containing 1 M urea for 30 min on ice. Rapid mixing of urea-loaded caveolae with buffer A containing 0.82 M sucrose (isosmotic with respect to urea-containing buffer) permitted measurement of urea permeability rates. Urea efflux in response to the chemical gradient was followed by water efflux with subsequent vesicle shrinkage. Proton permeabilities were measured by stopped-flow fluorometry as described (6, 17, 32). This assay utilized pH-dependent quenching of carboxyfluorescein on exposure of liposomes to isosmotic buffer at a reduced pH. The pH gradient was typically 0.4–0.6 pH units, which has been shown not to induce the formation of limiting potentials (8). Ammonia permeability was measured by rapid mixing of caveolae vesicles with buffer A containing 20 mM NH₄Cl and pH adjusted to 6.8. Before the experiment vesicles were prequillibrated in buffer A, pH 6.8. At this lower pH, the NH₄⁺:NH₃⁺ ratio is higher, increasing the gradient for NH₃, which may be calculated from the Henderson-Hasselbalch equation. To control for fluorescence changes unrelated to volume or pH, vesicles were routinely mixed with control buffer. These buffer shots were typically flat, indicating no artefactual changes in fluorescence and were subtracted from experimental curves to obtain the final kinetic traces.

Activation energy coefficient calculation. Water permeabilities were measured at five different temperatures between 4° and 30°C. Activation energy (Eₐ) was calculated from the slope of the natural log of the rate plotted against 1/T, multiplied by the gas constant, R.

Determination of liposome and vesicle size. Size distributions were determined by quasi-elastic light scattering with the use of a DynaPro LSR particle sizer and DYNAMICS data collection and analysis software (Protein Solutions, Buckinghamshire, UK).

Immunoblotting for caveolin-1 and AQP1. Equal volumes (40 μl) of gradient fractions taken from the first and second Optiprep gradients were run on SDS-PAGE using 10% precast minigels (Gradipore, French’s Forest, NSW, Australia) and then electrotransferred to Immuno-Blot PVDF membrane (Bio-Rad, Hercules, CA) at 250 mA for 1 h. Caveolin-1 was detected using polyclonal antisera against the NH₂-terminus (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:2,000 dilution and detected by ECL (Amersham Biosciences, Piscataway, NJ) using standard methods. Samples probed for AQP1 (20–50 μg total protein/lane) were run on 8–16% polyacrylamide gradient gels and immunoblotted with polyclonal AQP1 antibody (Chemicon International, Temecula, CA) at 1:2,000 dilution.

Electron microscopy. CEM were prepared as described above and adhered to grids by placing a 10-μl drop of enriched caveolae fraction on the surface of butvar-coated nickel grids for 15 min at room temperature. The following incubations were performed by inverting the grids on 200-μl drops of the appropriate solution, spotted on sheets of parafilm. The fraction was fixed with 4% (wt/vol) paraformaldehyde in 100 mM cacodylate, pH 7.4, rinsed with PBS three times, and then incubated three times for 5 min with 0.15% (wt/vol) glycine and 0.5% (wt/vol) BSA dissolved in PBS (buffer A). The sample was then incubated with caveolin-1 antibody diluted 1:50 in buffer A for 60 min at room temperature, washed three times for 5 min...
with buffer A, and incubated with protein A-10 nm colloidal gold (purchased from Dr. Jan Slot, Utrecht University, The Netherlands) diluted in the same buffer for 30 min at room temperature. The sections were further washed three times for 5 min with buffer A, washed with PBS, fixed with 2.5% (vol/vol) glutaraldehyde (in PBS) for 5 min, rinsed with PBS, then water, treated with 2% (wt/vol) neutral uranyl acetate, rinsed with water, stained with 4% (wt/vol) aqueous uranyl acetate, and then embedded in 1.2% (wt/vol) methylcellulose containing 0.1% (wt/vol) uranyl acetate. Sections were viewed at 80–100 kV with an electron microscope (model 110C, Jeol). Images were captured on film, scanned on a Saphir Ultra II scanner (Linotype-Hell, Eschborn, Germany), contrast corrected with Photoshop version 7.0 (Adobe, Mountain View, CA), and assembled in Freehand version 10.0 (Macromedia, San Francisco, CA).

Immunofluorescent labeling of lung tissue and image acquisition. Freshly excised rat lung tissue was cut in small blocks and frozen in liquid nitrogen. The tissue blocks were placed in optimum cutting temperature compound (Tissue-Tek, Torrance, CA) and rapidly frozen in molds placed on dry ice blocks. Frozen tissue blocks were stored at −80°C. Sections were cut at a thickness of 4 μM with the use of a cryostat machine (model CM1900, Leica, Bannockburn, IL), collected on Fisherbrand Superfrost/Plus slides (Fisher, Pittsburgh, PA), and then dehydrated by incubating the slides for 30 s at room temperature. The tissue sections were fixed using a pH-shift protocol as described previously (1). The nonreacted paraformaldehyde was quenched and the cells permeabilized with PBS containing 20 mM glycine, pH 8.0, 75 mM ammonium chloride, and 0.1% (vol/vol) Triton X-100 for 10–15 min at room temperature. The cells were washed with block solution [PBS containing 0.7% (wt/vol) fish skin gelatin and 0.025% (wt/vol) saponin] and then incubated in block solution containing 10% (vol/vol) newborn calf serum for 15 min at ambient temperature. Tissue was subsequently washed in block solution three times over 15 min and then incubated for 60–120 min at ambient temperature with primary antibody diluted in block solution. After incubation with the primary antibody, the tissue was washed three times with block solution for 15 min. The tissue was then incubated with the secondary antibody for 60–120 min at ambient temperature, and then washed three times with block solution over a 15-min time period. After three washes with PBS, the tissue was postfixed for 10–15 min in 4% (wt/vol) paraformaldehyde in 100 mM sodium cacodylate, pH 7.4. After a final wash with PBS, the coverslips were placed over the stained cryosections using p-diaminobenzidine-containing mounting medium.

Imaging was performed on a TCS-SL confocal microscope equipped with argon, and green and red helium-neon lasers (Leica, Deerfield, IL). The images were acquired by sequential scanning with a 0.5–1.0 μm step size. The images (512 × 512 pixels) were saved as Tiff files. The contrast level of the final images was adjusted with Adobe Photoshop, (Macromedia, San Francisco, CA).

RESULTS

The excised lungs were minced and homogenized, and after slow-speed centrifugation (1,000 g), a postnuclear supernatant was loaded and centrifuged on a Percoll gradient. The crude plasma membrane fraction from the Percoll gradient was then sonicated to cause the caveolae to pinch off from the bulk membrane and was then “floated” on two consecutive Optiprep gradients to yield a highly enriched caveolin-1 positive fraction. Figure 1A shows an immunoblot analysis of caveolin-1 in fractions from the initial Optiprep gradient. Total cellular caveolin-1 is found with a range of different buoyant densities and can be seen distributed throughout the gradient. Figure 1B shows the protein concentration profile of the first Optiprep gradient (Fig. 1A) and demonstrates that the majority of protein sediments in fractions with a higher density (i.e., fractions 6–14). Fractions 1–5 were run on SDS-PAGE and immunoblotted with caveolin-1 antibody. The arrow shows caveolin-1. B: protein concentrations of fractions shown in A. C: fractions 1–5 from (A) were recentrifuged on a second discontinuous Optiprep gradient. Equal volumes of fractions from the second Optiprep were run on SDS-PAGE and immunoblotted for caveolin.

Fig. 1. Purification of caveolae on Optiprep gradients. Membranes from homogenized rat lung were centrifuged on a 20%-10% continuous Optiprep gradient. A: fractions are removed from the top (fraction 1) and then equal volumes are run on SDS-PAGE and immunoblotted with caveolin-1 antibody. The arrow shows caveolin-1. B: protein concentrations of fractions shown in A. C: fractions 1–5 from (A) were recentrifuged on a second discontinuous Optiprep gradient. Equal volumes of fractions from the second Optiprep were run on SDS-PAGE and immunoblotted for caveolin.

only 5% of vesicles typically labeled. Others have noted similar inefficiencies (10). Our preparation with slightly higher immunogold labeling is thus likely to represent an enriched population of caveolae, which antibody binding underestimates.

The kinetics of water permeation across CEM were measured by stopped-flow fluorometry (Fig. 3A, control). Water efflux in response to a rapidly imposed twofold osmotic gradient is reflected by vesicle shrinkage to one-half the original volume. Traces shown are an average of 8–10 individual curves and fitted to a single exponential curve (superimposed). The \( P_f \) coefficient of CEM was 2.85 \( \pm 0.41 \times 10^{-3} \) cm/s (means \( \pm \) SE, \( n = 8 \) preparations), which is 5- to 10-fold higher than other membranes, both artificial and naturally occurring, known to contain high levels of cholesterol and sphingomyelin. To examine the influence of cholesterol in these membranes, CEM were treated with the cholesterol-sequestering drug methyl-\( \beta \)-cyclodextrin (10 mM for 30 min). Removal of cholesterol increased water permeability threefold (Fig. 3A; cyclodextrin), indicating that the permeability properties of these membranes is due in part to the presence of high levels of cholesterol.

The relatively high permeability of these membranes was surprising because we have previously shown that high concentrations of cholesterol and sphingolipids drastically reduce water and solute permeability by increasing lipid packing (14, 16). We therefore investigated whether aquaporin water channels could be contributing to the permeability. Schnitzer and Oh (34) presented evidence that caveolae isolated from rat lung endothelia contained AQP1 and hypothesized that fluid balance in the lung was regulated by the presence of aquaporins, 70% of which were localized to the caveolae on the luminal surface of blood vessels. Because our caveolar preparation was likely to contain a high proportion of endothelia-derived caveolae, we tested the effect of \( \text{HgCl}_2 \), an inhibitor of aquaporin-mediated water permeability. Figure 3B shows that 30-min incubation of vesicles with 1 mM \( \text{HgCl}_2 \) had no effect on water flux kinetics, demonstrating the absence of significant aquaporin-mediated water transport. \( \text{HgCl}_2 \) at 0.1 mM yielded the same result (data not shown).
The $E_a$ for water transport across caveolae was determined by measuring flux kinetics over a range of temperatures. $E_a$ was found to be high at 19.4 kcal/mol (mean of two experiments), further indicating that aquaporin water channels are unlikely to be present at significant levels in these organelles. However, immunoblot analysis of lung homogenate (H; 50 µg protein), postnuclear supernatant (P; 50 µg protein) and caveolae (C; 30 µg protein) demonstrated the presence of AQP1 in our vesicle preparation (Fig. 4). Comparison with 50 µg rat red blood cell (RBC) membranes (obtained by osmotic lysis and membrane pelleting) run as a positive control on the same gel, revealed that levels of AQP1 in our caveolae preparation are fourfold higher than in the homogenate, but only one-fifth of the level found in RBCs (after quantitation by densitometry and normalization for protein loading).

To further explore the question of whether AQP1 is primarily localized to caveolae in the lung, we performed double immunolabeling of intact lung tissue with antisera to both proteins (Fig. 5). The morphological data presented in Fig. 5 suggest that there is some colocalization between caveolin-1 (green) and AQP1 (red) in lung endothelial cells, as indicated by the sparse appearance of punctuate yellow spots. However, it is apparent that these areas represent only a small fraction of the total labeling for each protein and suggests that whereas some caveolae may be AQP1 positive, the majority do not possess water channels. This result is in agreement with our functional data (Fig. 3B) showing no influence of water channels on caveolae permeability and also our immunoblotting results showing a modest degree of enrichment (Fig. 4) for AQP1 in our CEM. These combined findings suggest that AQP1 is present at levels which are too low to significantly affect caveolae water permeability.

CEM permeability to solutes, urea, NH$_3$, and protons was investigated. Stopped-flow tracings are shown in Fig. 6 and illustrate widely varying time courses for these very different permeating species. The respective permeability coefficients for urea, NH$_3$, and protons were $8.85 \pm 2.40 \times 10^{-7}$, $6.84 \pm 1.03 \times 10^{-2}$, and $8.84 \pm 3.06 \times 10^{-2}$ cm/s (means ± SE, $n = 4$), which are relatively high also.

To understand the physical basis for higher-than-expected osmotic water permeability of CEM, we performed experiments on artificial membranes that had lipid compositions putatively similar to that found in the inner leaflet and the outer leaflet of caveolae. The experimental rationale was that in studying membranes devoid of proteins we could indirectly assess the contribution of those proteins to caveolae permeability. We have used this strategy previously and shown that symmetrical liposomal membranes modeling the inner and outer leaflet of the apical membrane of Madin-Darby canine kidney (MDCK) cells successfully recapitulate the permeability properties of the native membrane (14, 15). The basis for designing caveolae liposomes was provided by the in-depth compositional analysis provided in (27). Using electrospray ionization mass spectrometry, Pike et al. (27) quantitatively analyzed the lipid composition of caveolae and found high levels of arachidonic acid-containing plasmenylethanolamine in addition to the expected enrichment of cholesterol and sphingomyelin compared with plasma membrane.

Because we were constructing liposomes with “inner leaflet” lipids and liposomes with “outer leaflet” lipids, it was necessary to account for the asymmetric distribution of lipids in the bilayer. We therefore assumed that all of the phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol species were present on the inner leaflet and all the phosphatidylincholine and sphingomyelin was present in the outer leaflet. These assumptions are supported by a reasonable body of experimental evidence although it should be noted that most are studies performed on polarized epithelial cells (14, 36, 40, 41). In brush border membranes from rabbit intestine for example, it was shown that phosphatidylethanolamine was largely inaccessible to reagents from the outside (3). A similar accessibility study of rabbit kidney brush border membranes revealed that phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol were localized to the inner leaflet, whereas sphingomyelin (SM) was exoplasmic (43). In MDCK cells, a fluorescent ceramide analogue, which was taken up and converted to SM and glycosphingolipid in the Golgi, was completely extractable by BSA from the apical side, demonstrating a preferential localization in the exoplasmic leaflet.

**Fig. 4.** Immunoblot analysis for aquaporin-1 (AQP1). H, 50-µg homogenate; P, 50-µg post nuclear supernatant; C, 30-µg caveolae; RBC, 50-µg red blood cell membrane.

**Fig. 5.** Immunolocalization of AQP1 (red) and caveolin-1 (green) in rat lung. Frozen, fixed lung tissue (4 µm thick) was immunostained with antisera to AQP1 (red) and caveolin-1 (green) and imaged with confocal microscopy, as described in MATERIALS AND METHODS. Blue areas are nuclei and yellow indicates colocalization of proteins. The white square (middle) has been magnified and shown at bottom left. Arrows indicate areas of red and green overlap. Lungs from two animals were stained and imaged. Both exhibited the same staining patterns.
The single leaflet permeabilities of symmetrical liposomes and permeability and more importantly, the permeability across a calculate the contribution of an individual leaflet to the water therefore it cannot be assumed that the process obeys the concept of additive resistances that we know applies to water and solute permeation (13, 15, 23). However, the similarity of values for leaflets of differing composition as well as to the permeability of native caveolar membrane suggest that the caveolae we have isolated do not have a significant proton pump activity associated with them under the conditions of our assay and that the presence of integral membrane proteins such as caveolin does not significantly affect passive permeability to protons.

**DISCUSSION**

Caveolae are a compositionally unique, dynamic cellular organelle with a diverse array of potential functions. Despite much progress in the past decade, the role of caveolae remains unclear and appears to be cell and tissue type specific. To study the physical properties of caveolae in more detail, we utilized a non-detergent-based isolation procedure for obtaining a highly enriched caveolae membrane preparation from the rat

**Table 1. Leaflet composition by lipid class**

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Outer Leaflet Lipids, mol%</th>
<th>Inner Leaflet Lipids, mol%</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE</td>
<td>26</td>
<td>56.4</td>
</tr>
<tr>
<td>SM</td>
<td>30.3</td>
<td>13.8</td>
</tr>
<tr>
<td>PS</td>
<td>4.8</td>
<td>18</td>
</tr>
<tr>
<td>PA</td>
<td>1.8</td>
<td></td>
</tr>
</tbody>
</table>

PE, plasmeneylethanolamine; PI, phosphatidylinositol; SM, sphingomyelin; PS, phosphatidylserine; PA, phosphatic acid; PC, phosphatidylcholine. adding their reciprocals (see Ref. 23) results in a permeability coefficient for a model deproteinated caveolar membrane of 1.58 ± 1.1 × 10^-3 cm/s, which is close to the value obtained for native caveolae (2.85 ± 0.41 × 10^-3 cm/s).

Inner and outer leaflet permeabilities to protons were measured at 4.42 ± 1.0 × 10^-2 and 6.55 ± 2.4 × 10^-2 cm/s respectively (data not shown). These values are quite close to those obtained for native caveolae, which was 8.84 × 10^-2 cm/s. It is not possible to calculate the proton permeability of a combined inner and outer leaflet membrane because the mechanism by which protons permeate is not entirely clear and therefore it cannot be assumed that the process obeys the concept of additive resistances that we know applies to water and solute permeation (13, 15, 23). However, the similarity of values for leaflets of differing composition as well as to the permeability of native caveolar membrane suggest that the caveolae we have isolated do not have a significant proton pump activity associated with them under the conditions of our assay and that the presence of integral membrane proteins such as caveolin does not significantly affect passive permeability to protons.

**Table 2. Fatty acyl chain composition of liposomes**

<table>
<thead>
<tr>
<th>Fatty Acids</th>
<th>Outer Leaflet</th>
<th>Inner Leaflet</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0–16:0</td>
<td>20.30%</td>
<td>16.00%</td>
</tr>
<tr>
<td>16:0–18:1</td>
<td>9.40%</td>
<td>6.10%</td>
</tr>
<tr>
<td>16:1–18:1</td>
<td>5.30%</td>
<td>5.40%</td>
</tr>
<tr>
<td>18:0–18:2</td>
<td>1.70%</td>
<td>2.30%</td>
</tr>
<tr>
<td>18:1–18:1</td>
<td>23.70%</td>
<td>24.10%</td>
</tr>
<tr>
<td>16:0–18:1</td>
<td>3.80%</td>
<td>4.50%</td>
</tr>
<tr>
<td>18:1–18:1</td>
<td>3.80%</td>
<td>5.40%</td>
</tr>
<tr>
<td>18:1–20:4</td>
<td>2.30%</td>
<td>2.30%</td>
</tr>
<tr>
<td>Brain SM</td>
<td>PE</td>
<td>PA</td>
</tr>
</tbody>
</table>

Letters in parentheses refer to the following Avanti catalogue numbers: a, 850355; b, 850457; c, 850375; d, 850469; e, 860032; f, 850758; g, 850752; h, 850806; i, 850804; j, 852757; k, 852801; l, 840857; m, 840063; n, 840044

Fig. 6. Stopped-flow recordings showing permeation kinetics for urea (A), NH₃ (B), and protons (C). Data shown are representative of three preparations.

Stopped-flow tracings showing the different kinetics of water flux through the two membranes is shown in Fig. 7. The Pᵣ of inner leaflet and outer leaflet liposomes was dramatically different, with the inner leaflet having much faster shrinkage kinetics in response to an osmotic gradient. Figure 7, inset, shows the inner leaflet curve in more detail. Pᵣ for the outer and inner leaflet were calculated to be 8.84 ± 3.1 × 10^-4 and 7.42 ± 1.1 × 10^-3 cm/s, respectively, an eight-fold difference. We (13, 14, 23) have shown previously that each leaflet offers an independent resistance to permeation, thus allowing us to calculate the contribution of an individual leaflet to the water permeability and more importantly, the permeability across a bilayer composed of different leaflets. Therefore, calculating the single leaflet permeabilities of symmetrical liposomes and...
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Fig. 7. Stopped-flow recordings showing permeation kinetics for water across liposomes with compositions designed to mimic the inner and outer leaflet of caveolae. Inset, expanded view of inner leaflet water permeation kinetics.

It is of interest that even investigations employing immunosolation of caveolae came to very different conclusions regarding the protein composition of the isolated membranes and in particular the presence or absence of signal transduction molecules such as G proteins and endothelial nitric oxide synthase (24, 39). It appears that differences in methodology for preparing membrane fractions before immune separation may have contributed to the different findings. These studies emphasize some of the technical difficulties in isolating caveolae and have led to a certain degree of confusion in the literature regarding measures of purification and precise compositional definition.

An advantage of using the rat lung as a source of caveolae is that nearly all of the caveolae detectable by immunogold localization in situ are associated with the endothelial and epithelial surfaces rather than with intracellular structures such as the Golgi, thus resulting in a population, which is more homogenous in its protein and lipid composition (24). The caveolar vesicles we obtained were slightly larger (~150 nm mean diameter) than is normally associated with the classic “flask” shaped structure in vivo (50–120 nm). However, it is becoming apparent that caveolae are a morphologically heterogeneous organelle due to a range of membrane trafficking dynamics, which include endocytosis, potocytosis, and tubule formation, and cannot be considered just simple invaginations of the membrane. Endothelial cells, for example, are rich in vesicles and tubules that do not fit the above rigid criteria (31). Stan et al. (39) noted that there was a difference in the size distribution of immunosolated caveolae compared with caveolae in situ and speculated that size differences arose during membrane fragmentation and rescaling processes on sonication.

The osmotic water permeability of CEM at $2.9 \times 10^{-3}$ cm/s was higher than we had expected because enrichment of lipids such as sphingomyelin and cholesterol are known to form ordered microdomains with low fluidity and therefore low permeability. Indeed, caveolae have been shown to contain 225% as much cholesterol as is found in the plasma membrane (constituting 33.5% of the total lipid complement) and 46% more sphingomyelin (27). Cyclodextrin treatment of the membranes resulted in an increase in permeability, demonstrating that the presence of cholesterol does indeed contribute somewhat to higher lipid packing densities and the buoyant density properties, which allow for its purification. However, apical membranes from the stomach, bladder, and MDCK cells, which are rich in these lipids, typically exhibit permeabilities on the order of $2 - 6 \times 10^{-4}$ cm/s (6, 17, 18), which are ~10 times lower.

What contributes to this rather high water permeability? We tested for the presence of AQP1, which has been shown to be concentrated in the caveolae of endothelial cells (34). Caveolae from our preparation, however, exhibited high-activation energy for water transport and no inhibition of water flux on treatment with HgCl$_2$. By contrast, we were able to demonstrate the presence of AQP1 by immunoblot analysis. To gain further insights into this apparent discrepancy, we examined the cellular distribution of caveolin-1 and AQP1 in situ by double immunostaining. The results clearly showed that there were some areas of overlap in the cellular localization of the two proteins, but that >90% did not overlap. This finding is consistent with our observations of modest AQP1 enrichment in our CEM (by immunoblotting) and with the lack of functional water transport attributable to water channels. Schnitzer and Oh (34) concluded that 70% of endothelial AQP1 was localized in caveolae based on immunoblotting of rat lung caveolae purified from silica-coated plasma membranes. Our in situ staining data does not support such a conclusion. It appears there is some AQP1 in caveolin-positive regions of the membrane but that the majority is differentially segregated. It is likely that the lack of functionally detectable AQP1-mediated water transport is because AQP1 is present at levels which are too low to be functionally relevant in the isolated caveolae vesicle. The relative band densities shown in Fig. 4 demonstrate that AQP1 is present at only one-fifth the level seen in RBCs. This is likely to be sufficiently low as to not significantly affect the activation energy required for water transport and to be functionally undetectable in our assay. Mathai et al. (21) demonstrated a 70–75% reduction in osmotic water permeability in human red blood cells from Colton-Null heterozygotes with 50% of normal AQP1 levels. The lack of linear dose response to protein level, suggests that CEM with AQP1 at
~20% of the density found in RBCs will not significantly contribute to membrane permeability.

In the absence of a mercury-inhibitable water channel, we were interested to know whether the presence of embedded caveolin protein was contributing to the relatively high permeability we observed. On the basis of its known lipid composition, we expected P_f of caveolae to be low. Because this was not the case, it suggested that the insertion of multiple hairpin peptide segments from oligomerized caveolin-1 and -2 could disrupt the normally tight packing arrangement of lipids. By creating more free volume area between lipids, water could conceivably permeate more freely.

Because ablation of caveolin expression in cell culture or in knockout animals results in loss of morphologically distinct caveolae (9, 30), and because there are no useful approaches for removing caveolin and other integral membrane proteins from isolated caveolar membranes without destroying important elements of membrane substructure, it was necessary to utilize a membrane reconstitution strategy to address the role of proteins in influencing caveolae permeability.

Our membrane reconstitution experiments showed very similar water permeability in liposomes designed to mimic caveolae membrane structure in the absence of proteins. Because of the close concordance of values obtained in native CEM and in reconstituted liposomal membranes we conclude that membrane perturbations may contribute in a modest manner, but that other structural features, notably acyl chain unsaturation, are more important. Pike et al. (27) noted extensive enrichment of 20:4 (arachidonic acid) acylation and estimated that arachidonic acid-containing plasmalogenethanolamines represented up to a half of all cytosolic leaflet lipids. This degree of unsaturation will lead to a more disordered hydrophobic region within the bilayer and hence more free volume space for water and other solutes to occupy.

An interesting corollary of our conclusion that caveolae lipids are not in a classic liquid-ordered state pertains to the apparent exclusion of glycosylphosphatidylinositol-anchored proteins from caveolae (20, 25, 33). Because the glycosylphosphatidylinositol anchor is thought to be responsible for the localization of these proteins in lipid rafts due to its preferential miscibility in highly ordered domains, their exclusion as a class of molecules from caveolae might arise precisely because the lipids are not so tightly packed. Conversely, proteins found in caveolae and not lipid rafts, such as platelet-derived growth factor receptor, tyrosine kinases, and the heterotrimeric G protein, G_q, may partition into caveolae due to the particular physical properties exhibited by the caveolar bilayer and/or through interactions with caveolin (25).

The permeability values obtained for urea and NH_3 are also consistent with P_f in terms of passive permeability observed in a range of other membrane systems (6, 7, 18, 28, 29, 32). This suggests the absence of specific transporters for either urea or NH_3 in caveolae. Proton permeability is also consistent with our measurements across pure lipid membranes that mimic caveolae structure, suggesting that there is unlikely to be a proton transport activity present under our experimental conditions, which employs no added ATP.

Our initial predictions that caveolae would be low-permeability membrane microdomains proved to be incorrect. Despite being significantly enriched for cholesterol over bulk plasma membrane, these membranes retain a relatively high permeability to water and solutes. It is intriguing to note that sphingomyelin is not as enriched as cholesterol in caveolae (27) because cholesterol is known to interact through hydrogen bonding with this lipid (4) and is thought to be concentrated in the outer leaflet. Because caveolins are an important component in the cellular cholesterol trafficking machinery and cholesterol appears to use caveolae as a “docking” point before efflux from the cell (31), it is possible that a proportion of caveolae-localized cholesterol is sequestered differently; perhaps in the cytosolic leaflet or bound to caveolin. As such, some caveolae-localized cholesterol may not be free to display the typical membrane condensing properties for which it is well known.

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