Isolation and characterization of the Xenopus oocyte plasma membrane: a new method for studying activity of water and solute transporters

Warren G. Hill,1 Nicole M. Southern,1 Bryce Maclver,1 Elizabeth Potter,2 Gerard Apodaca,1 Craig P. Smith,2 and Mark L. Zeidel1
1Laboratory of Epithelial Cell Biology, Renal-Electrolyte Division, Department of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania; and 2Department of Molecular Cell Physiology, Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom

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Hill, Warren G., Nicole M. Southern, Bryce Maclver, Elizabeth Potter, Gerard Apodaca, Craig P. Smith, and Mark L. Zeidel. Isolation and characterization of the Xenopus oocyte plasma membrane: a new method for studying activity of water and solute transporters. Am J Physiol Renal Physiol 289: F217–F224, 2005. First published March 1, 2005; doi:10.1152/ajprenal.00022.2005.—The intact Xenopus laevis oocyte is a useful model system for studying expressed water and solute transporters but suffers from a number of limitations, most notably large unstirred layers and other intracellular diffusion barriers. To overcome these, we have developed a method for isolating plasma membrane vesicles from oocytes. This approach facilitates more precise control of the intravesicular environment and virtually eliminates the problem of unstirred layers in kinetic experiments. The isolation procedure results in 50.6-fold enrichment of the plasma membrane marker alkaline phosphodiesterase compared with the homogenate. Markers of late endosomes/lysosomes and mitochondria were not enriched, and the endoplasmic reticulum was enriched only modestly. Permeabilities of native plasma membrane to water and urea were 8.1 × 10−4 and 5.6 × 10−7 cm/s, respectively, values that are sufficiently low to classify them as barrier membranes. Phospholipid analysis by mass spectrometry showed the membrane, not including cholesterol, to be rich in phosphatidylcholine (35.8 mole percent), sphingomyelin (25.8 mole percent), and phosphatidylinositol (6.8 mole percent). Cholesterol concentration was 20.7 mole percent. Membrane vesicles isolated from oocytes expressing aquaporin-1 exhibited fourfold higher water permeability in stopped-flow experiments. Oocytes expressing mouse urea transporter A3 (UT-A3) exhibited 7.5-fold faster phloretin-inhibitable urea transport compared with water-injected controls. There was no difference in water permeability between these membrane vesicles, suggesting that UT-A3 is not a water carrier. In conclusion, we describe an improved method for the isolation of the oocyte plasma membrane that will allow the study of water and solute transport kinetics as well as substrate selectivity in heterologously expressed proteins.

DEFINING THE FUNCTION OF PROTEINS requires that their activity be measured under controlled conditions in vitro. Unlike soluble proteins, membrane proteins, particularly transporters, must be isolated within membrane fractions to be functional. Multiple expression systems have been developed for transporters. These include bacterial spheroplasts, yeast sec6 vesicles, membranes isolated from baculovirus-infected insect sf-9 cells, as well as reconstitution of purified proteins in proteoliposomes. In each of these vesicle systems, the composition of the solutions on either side of the transporter can be tightly controlled and, using stopped-flow or rapid filtration techniques, unstirred layers can be minimized. Unfortunately, however, many proteins cannot be successfully expressed in any of these systems.

By contrast, Xenopus laevis oocytes have proven to be remarkably effective for expression of a wide variety of transporter proteins. In the case of ion channels, detailed functional studies of expressed proteins can be performed using two-electrode voltage-clamp or patch-clamp techniques. For proteins involved in electroneutral transport or flux of nonionic substances such as water or urea, electrode methods will not work. While isotopic fluxes or osmotically induced swelling in intact oocytes permits some measurement of function, the inability to control the composition of the interior of the oocyte limits the functional data that can be obtained. It is important to recognize that the intact oocyte is an imperfect system for making measurements of diffusive transport phenomena due to large unstirred layers on both sides of the limiting membrane. In addition, the internal milieu of the oocyte represents a viscous, heterogenous medium replete with diffusion barriers and areas that may be osmotically insensitive, e.g., the nucleus. Intracellular pH measurements related to CO2 or NH3 fluxes are also similarly compromised by multiple unmeasurable diffusion barriers within the cell.

Our aim in this study was to gain the advantages of the oocyte as an expression system, as well as the functional resolution of vesicle systems, by developing a method to isolate native X. laevis oocyte plasma membranes and plasma membranes expressing transporter proteins and determine whether these vesicles are suitable for the assessment of heterologously expressed proteins. We successfully demonstrate the utility of this method for the study of transporters by demonstrating the use of aquaporin-1 (AQP1) and mouse urea transporter UT-A3.

One reason the oocyte has proven to be so useful for the study of water channels is that it has a remarkable capacity to resist volume expansion on osmotic challenge. When oocytes are placed in 10 mosmol/kgH2O buffer, 40% remain unburst after 2 h (14), demonstrating that the plasma membrane and potentially its associated structural components, i.e., the cytoskeleton and vitelline membrane, present an extremely effective barrier to the passive diffusion of water. Many investiga-

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tors have measured the osmotic ($P_t$) water permeability of whole oocytes, often in the context of comparisons with aquaporin (AQP)-expressing cells, and values reached in all of these studies show good agreement in the range of $0.6-1.5 \times 10^{-3}$ cm/s (1, 3, 4, 12, 15, 20, 24, 25). Isolation of the plasma membrane allowed us to test its permeability properties and confirm that its resistance to osmotic swelling was an intrinsic property of the membrane alone.

Low-permeability membranes are important in various tissues for the maintenance of osmotic and chemical gradients. In mammals, examples may be found in the urinary bladder, the stomach, and in specific segments of the nephron where impermeable epithelial apical membranes are required for the urine concentrating mechanism. These membranes not only lack AQP water channels but have unique lipids that appear to result in tighter packing or higher membrane order, thereby reducing defects through which water and solutes can enter and diffuse. How cells synthesize barrier membranes and how the presence or absence of particular lipids affects barrier function are largely unknown. We propose that the plasma membrane of the oocyte can also serve as a useful model for barrier membranes and that, having established a method for isolating it, we can take advantage of the oocyte’s size and expression capabilities to explore barrier membrane biogenesis.

**METHODS**

**Materials.** Collagenase and trypsin inhibitor were from Sigma (St. Louis, MO). Antibodies to Rap1, manganese (Mn) SOD, and calnexin were obtained from Stressgen Biotechnologies (Victoria, BC). AQP1 was from Chemicon International (Temecula, CA), and protein disulide isomerase (PDI) antibody was from BD Transduction Laboratories (San Diego, CA).

**Harvesting oocytes.** *X. laevis* frogs (Xenopus Express, Plant City, FL) were anesthetized in 1 liter of 0.5% (wt/vol) 3-aminobenzoic acid (Fluka, St. Louis, MO). Antibodies to Rap1, manganese (Mn) SOD, and calnexin were obtained from Stressgen Biotechnologies (Victoria, BC). AQP1 antibody was from Chemicon International (Temecula, CA), and protein disulide isomerase (PDI) antibody was from BD Transduction Laboratories (San Diego, CA).

**Expression of mouse UT-A3.** Mouse UT-A3 (GenBank accession no. AF258602) (7) in pPT7S transcription vector was linearized using EcoR1, and cRNA was prepared using the T7 mRNA machine kit from Ambion (Austin, TX). Oocytes were injected with 10 ng RNA and incubated at 18°C for 3 days. Expression was confirmed by placing oocytes in distilled water and monitoring the swelling and bursting times before membrane preparation.

**Expression of human AQP1.** The human AQP1 X. laevis expression vector (no. 99538) was acquired from ATCC (Manassas, VA). Capped cRNA was prepared with an mMessage mMachine kit from Ambion (Austin, TX). Oocytes were injected with 10 ng RNA and incubated at 18°C for 3 days. Expression was confirmed by placing oocytes in distilled water and monitoring the swelling and bursting times before membrane preparation.

**Assessment of swelling kinetics.** Oocytes were imaged on a Nikon inverted microscope using a ×2 objective, and images were captured with a Hamamatsu CCD camera and Simple PCI software. Images were acquired every 15 s, and image stacks were thresholded to black and white and analyzed for oocyte cross-sectional area using ImageJ software.

**Permeability assays.** Permeabilities were measured with stopped-flow fluorometry (10, 19). Water permeability was measured from the rate of shrinkage after exposure of membrane vesicles to a hypertonic solution with double the osmolality. Urea transport was measured in membrane vesicles preequilibrated for 20–30 min in OHB containing 1 M urea. Vesicles were then rapidly exposed to an osmotically balanced solution containing 500 mM urea. Urea efflux in response to the chemical gradient leads to vesicle shrinking. In some experiments, UT-A3 was inhibited by preincubation with 0.5 mM phloretin for 20–30 min.

**Analysis of lipids by mass spectrometry.** Lipids were extracted in chloroform:methanol (2:1, vol/vol) containing 0.2 mg/ml BHT overnight at 4°C under a nitrogen atmosphere. Three hundred microliters of 0.15 M NaCl were added to affect phase separation. The chloroform layer was removed and dried under a stream of nitrogen. Lipids were then analyzed by electrospray ionization tandem mass spectrometry either by direct infusion or after chromatographic enrichment on a Micromass Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, UK). Sheath flow was adjusted to 5 μl/min and consisted of methanol:chloroform (2:1, vol/vol). The electrospray probe was operated at a voltage differential of 3.5 kV in either the positive or negative ion mode. Mass spectra were obtained by scanning in the range of 400–950 m/z every 1.6 s and summed. Source temperature was maintained at 70°C. CID spectra were obtained by...
selecting the ion of interest and performing daughter ion scanning in Q3 at 400 amu/s using Ar gas in the collision chamber. The spectrometer was operated at unit resolution. A combination of daughter, parent, and neutral loss ion-scanning techniques was used to identify and quantitate the various lipid species.

**Immunoblotting.** Membrane samples were run on SDS-PAGE using 10% precast minigels (Gradipore, French’s Forest, NSW, Australia) and then electrotransferred to Immuno-Blot polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA) at 250 mA for 1 h. Antibodies to AQP1 (1:1,000), calnexin (1:2,000), Rap1 (1:200), MnSOD (1:500), and PDI (1:200) were incubated with electrotransferred samples and detected by ECL (Amersham Biosciences, Piscataway, NJ) using standard methods.

**Cholesterol assay.** Cholesterol was assayed on membranes extracted with hexane/isopropanol (3:2) and dried under nitrogen, using a Wako Cholesterol E kit (Wako Chemicals, Richmond, VA) according to the manufacturer’s instructions.

**Phospholipid quantitation.** Lipids were extracted from membranes with chloroform:methanol (1:2), and then the solvent was evaporated in a boiling water bath. Lipids to which 0.65 ml 70% perchloric acid had been added were heated to over 350°C in a glass-bead sterilizer for 30 min, and then inorganic phosphate was assayed by the method of Fiske and Subbarow (8). Briefly, samples were mixed with 3.3 ml water, 500 μl of 2.5% (wt/vol) ammonium molybdate, and 500 μl 10% (wt/vol) ascobic acid. Standards (0–50 μl, 439 μg/ml K2HPO4) were run in parallel. Tubes were incubated in a boiling water bath for 5 min, and then absorbance was read at 800 nm.

**Electron microscopy.** Freshly prepared oocyte membranes were mixed with 2× fixative containing 2.0% (vol/vol) glutaraldehyde and 2.0% (wt/vol) paraformaldehyde in 200 mM Na cacodylate, pH 7.4, and centrifuged in a RP45A rotor at 100,000 g in a RC M120EX ultracentrifuge (Sorvall) for 30 min at 4°C. The fixed pellet was removed from the bottom of the centrifuge tube, rinsed with 100 mM Na cacodylate, pH 7.4, buffer, cut into small pieces, and treated with 1% (wt/vol) OsO4 in 100 mM Na cacodylate, pH 7.4, buffer for 60 min at 4°C. After several water rinses, the samples were stained en bloc overnight with 0.5% uranyl acetate in water. Samples were dehydrated in a graded series of ethanol, embedded in the epoxy resin LX-112 (Ladd), and sectioned with a diamond knife (Diatome). Sections, silver to pale gold in color, were mounted on Butvar-coated copper grids, contrasted with uranyl acetate and lead citrate, and viewed at 80 kV in a Jeol 100 CX electron microscope. Images were captured on film, scanned on a Linotype-Hell Saphir Ultra II scanner (Eschborn), and contrast adjusted in Photoshop 7.0 (Adobe).

**Determination of membrane vesicle diameters.** Size distributions were determined by quasi-elastic light scattering using a DynaPro LSR particle sizer and DYNAMICS data collection and analysis software (Protein Solutions, Bucks, UK).

**RESULTS**

It was our goal to develop a system for purifying the plasma membrane of the oocyte so that expressed membrane transporters could be studied under more defined conditions and so that the barrier function of the native membrane could be examined. The method developed for purifying oocyte plasma membrane was based on modifications made to the membrane isolation procedure described elsewhere (17). Essentially, it consists of careful homogenization of oocytes, followed by a series of low-speed centrifugation steps to deplete the homogenate of large amounts of yolk protein and melanosomes and, finally, a single discontinuous sucrose gradient from which two membrane bands are harvested. It was noted from early experiments that more strokes with the Dounce homogenizer resulted in a high proportion of multilamellar vesicles, as seen by electron microscopy. Fewer strokes resulted in a preparation that was almost completely unilamellar and exhibited better homogeneity. Figure 1A shows electron micrographs of L-membranes that exhibited high buoyancy taken from near the top of the sucrose gradient, whereas Fig. 1B shows the heavy H-membrane fraction that sedimented at the 20%/50% sucrose interface. L-membranes show a population of vesicles that are reasonably uniform in size with few membrane fragments and very few multilamellar structures. Present in both images are large numbers of electron-dense granules that appear to be more concentrated in the H-membranes. Membranes in Fig. 1B also show evidence of contaminating intracellular organelles and a higher proportion of nonvesicular membranes.

![Fig. 1. Electron micrographs of light (L)-membranes (A) and heavy (H)-membranes (B). Scale bars = 1 μm.](http://ajprenal.physiology.org/ by 10.220.33.2 on October 29, 2017)
Marker enzymes were assayed for activity (Table 1) or by immunoblotting (Fig. 2) to determine the degree of enrichment or deenrichment of plasma membrane and intracellular membranes in the final purified fractions \((n = 4\) sets of oocytes). Alkaline PDE assays demonstrated that L-membranes were 50.6-fold enriched for plasma membrane compared with homogenate, whereas H-membranes were less enriched and only represented a small proportion (17%) of the plasma membrane recovered in the two fractions. Succinate dehydrogenase in L-membranes showed a small degree of enrichment (3-fold) compared with the homogenate. This was surprising given our immunoblotting result for MnSOD showing evidence of depurification of this mitochondrial marker (Fig. 2). It is possible that organelle damage could lead to inner membrane fragments partitioning with plasma membranes, whereas intact mitochondria would be expected to pellet. The lysosomal enzyme NAG (23) showed enrichment in both membrane fractions but was present at substantially higher levels in the H-membranes. Because lysosomes are a relatively dense organelle and NAG is a soluble enzyme, we believe the apparent enrichment in L-membranes may be the result of released enzyme rather than due to lysosomal contamination per se.

In Fig. 2, immunoblotting reveals relative levels of membrane markers in the homogenate, postnuclear supernatant, and L- and H-membranes from the sucrose gradient. Rap1, a member of the Ras family of small GTPases, is a late endosomal/lysosomal marker (18) and is absent from both L- and H-membranes. As Rap1 is a prenylated protein and therefore membrane associated, this result supports the suggestion that NAG contamination is due to organellar damage and soluble enzyme leakage. MnSOD appears to be completely absent from L-membranes, leading us to conclude that mitochondrial contamination is negligible. Calnexin and PDI are endoplasmic reticular markers and show enrichment in both H- and L-membranes and approximately equal concentrations. Quantitative densitometry revealed that there is 5.8- and 7.6-fold enrichment of calnexin and PDI, respectively, in L-membranes (mean, 6.7-fold), whereas H-membranes were 3.4- and 2.7-fold enriched for the same markers (mean 3.1-fold). Because the oocyte is known to possess extraordinarily high protein biosynthetic capabilities, perhaps it is not surprising that endoplasmic reticular membranes are difficult to eliminate completely. Compared with a 50-fold enrichment of plasma membrane, however, it is likely that endoplasmic reticular membranes are a minor component of the entire vesicle population.

Table 1. Marker enzyme enrichment in isolated membrane fractions compared with homogenate

<table>
<thead>
<tr>
<th>Marker enzyme (Fold-Enrichment)</th>
<th>Light Membranes</th>
<th>Heavy Membranes</th>
<th>% In Light:</th>
<th>% In Heavy:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphodiesterase (plasma membrane)</td>
<td>50.6 ± 3.3</td>
<td>10.3 ± 3.1</td>
<td>83:17</td>
<td></td>
</tr>
<tr>
<td>Succinate dehydrogenase (mitochondria)</td>
<td>3.3 ± 1.0</td>
<td>3.9 ± 0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-N-acetyl glucosaminidase (lysosomes)</td>
<td>12.8 ± 0.6</td>
<td>34.3 ± 6.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

We characterized the permeability properties of the L- and H-membrane fractions, because we were also interested in defining the biophysical properties of the oocyte plasma membrane in detail. Figure 3 shows the results of stopped-flow fluorometry experiments in which vesicles were rapidly exposed to either an osmotic or outward urea gradient. Under the conditions chosen, vesicles shrink. The kinetics of water and urea flux are reported by entrapped intravesicular CF, which self-quenches on vesicle shrinkage. Water flux kinetics (Fig. 3A) for H- and L-membranes reveal that L-membranes have a lower water permeability than H-membranes. The heavy fraction exhibits two distinct exponential decay rates (appropriate single and double exponential curves have been computer fitted to the raw data). This likely reflects the presence of at least two populations of membrane vesicles with very different permeability coefficients. \(P_t\) values from five separate oocyte membrane preparations are shown in the inset. Permeabilities were assigned to both the fast and slow component of the H-membrane curve. L-membranes have \(P_t = 8.07 \times 10^{-4}\) cm/s, whereas the fast and slow flux rates of H-membranes yielded values of \(2.83 \times 10^{-2}\) and \(8.58 \times 10^{-4}\) cm/s, respectively.

Interestingly, when the urea permeability of these membranes was examined (Fig. 3B), the double exponential decay

![Fig. 2. Immunoblotting of membrane fractions for organelle markers. RAP1 is a late endosomal/lysosomal marker, MnSOD is a mitochondrial marker, and calnexin and protein disulfide isomerase (PDI) are endoplasmic reticular markers. Lanes compare relative signal strength for equivalent protein loading (35 µg/lane) of oocyte homogenate (H), postnuclear supernatant (PN), and L- (LT) and H-membranes (HV).](http://ajprenal.physiology.org/)
was absent from H-membranes and a single permeability coefficient could describe the flux kinetics. Clearly, H-membranes were more permeable than the rather tighter L-membranes. The *inset* shows results from five sets of oocytes and reveals that urea permeability values for L- and H-membranes were $5.64 \times 10^{-7}$ and $5.44 \times 10^{-6}$ cm/s, respectively, a 10-fold difference.

A long-standing interest of our laboratory has been to understand the nature of the barrier function exhibited by some naturally occurring membranes and, more specifically, whether certain lipids or lipid combinations are responsible for reducing membrane permeability. Accordingly, we analyzed the lipid and acyl chain composition of L-membranes by mass spectrometry. The results, not including cholesterol, are shown in Table 2 and reveal that the oocyte plasma membrane is extremely rich in phosphatidylcholine (PC) and sphingomyelin (SM) species, which together account for 61.6% of total phospholipid. Separate assays of cholesterol and total phospholipid showed that cholesterol is present at mole percentages in L- and H-membranes of $20.6 \pm 1.2$ and $12.9 \pm 1.9$, respectively (means $\pm$ SE, $n = 5$ frogs). This is rather lower than is seen in the red blood cell, where membrane cholesterol constitutes 46 mole percent (16). Perhaps the most surprising result is the high level of phosphatidylinositol (PI).

To evaluate the usefulness of this isolation scheme for the study of transporters, we expressed AQP1 in oocytes. Figure 4 illustrates swelling kinetics of oocytes injected with water or with 10 ng of AQP1 cRNA. Oocytes were perfused with MBS and then switched to hypotonic MBS (diluted with water to 30%). The arrow in Fig. 4 indicates when hypotonic solution reaches the oocyte. AQP1-expressing oocytes swelled rapidly and burst, demonstrating the presence of functional water channels on the membrane surface.

Immunoblotting of membranes from AQP-expressing oocytes showed a dramatic enrichment of the 28-kDa band of AQP1 in the L-membranes (Fig. 5A) compared with the homogenate and postnuclear supernatant. Quantitative densitometry showed 103- and 52-fold enrichment of AQP1 in L- and H-membranes, respectively, compared with the homogenate. As expected, uninjected oocytes (lanes) showed no immunostaining. Red blood cells were run as a positive control and showed a majority of the protein as the 36-kDa glycosylated form of AQP1. Oocytes appear to make very little if any glycosylated AQP1.

L-membranes prepared from 300–400 oocytes exhibited faster water efflux kinetics in stopped-flow experiments (Fig. 5B) than control oocyte membranes. Control membranes were described well by a single exponential fit, where AQP-expressing membranes were fitted to a double exponential that included a fast component (Fig. 5B) and a slow component that had the same rate as control membranes. Data from three separate experiments and three membrane preparations are shown in the *inset*. Fast water efflux kinetics are AQP mediated and fourfold faster than passive permeability through the membrane.

Oocytes were also injected with 30 ng of RNA from UT-A3, the phloretin-inhibitable mouse urea transporter (7). Membranes prepared from these oocytes exhibited markedly elevated urea flux kinetics compared with water-injected control membranes (Fig. 6). Like AQP-expressing membranes, the kinetics of urea flux were best fitted by a double exponential (labeled in the *inset* bar graph as UT-fast and UT-slow). The fast rate was 7.5-fold higher than the control (Fig. 6, *inset*), and the slow rate was not different from control and therefore consistent with passive urea diffusion through the membrane. Despite the apparent variability in urea transport rate (Fig. 6, *inset*), the differences in two experiments between UT- and

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**Fig. 3.** Water and urea permeability of L- and H-membranes. Curves show stopped-flow tracings of vesicle shrinkage kinetics (average of 8–10 curves) normalized to relative volume. *A*: water permeability of light and heavy membranes. *B*: urea permeability ($P_{\text{urea}}$) of light and heavy membranes. *Insets*: calculated permeability coefficients ($P_f$) as means $\pm$ SE of 5 experiments. Heavy-fast and heavy-slow, permeabilities associated with 1st and 2nd exponential rates fitted to the curve.

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A long-standing interest of our laboratory has been to understand the nature of the barrier function exhibited by some naturally occurring membranes and, more specifically, whether certain lipids or lipid combinations are responsible for reducing membrane permeability. Accordingly, we analyzed the lipid and acyl chain composition of L-membranes by mass spectrometry. The results, not including cholesterol, are shown in Table 2 and reveal that the oocyte plasma membrane is extremely rich in phosphatidylcholine (PC) and sphingomyelin (SM) species, which together account for 61.6% of total phospholipid. Separate assays of cholesterol and total phospholipid showed that cholesterol is present at mole percentages in L- and H-membranes of $20.6 \pm 1.2$ and $12.9 \pm 1.9$, respectively (means $\pm$ SE, $n = 5$ frogs). This is rather lower than is seen in the red blood cell, where membrane cholesterol constitutes 46 mole percent (16). Perhaps the most surprising result is the high level of phosphatidylinositol (PI).
water-injected membranes were consistent at 7.7- and 6.9-fold, respectively. Phloretin effectively inhibited urea transport activity by 75%, demonstrating the utility of this approach for studying transporter kinetics and the pharmacological profile. 

\[ P_f \] of UT-A3-expressing membranes was the same as control membranes, demonstrating no significant water permeability associated with this transporter (not shown).

**DISCUSSION**

Development of a simple method for purifying oocyte plasma membrane in quantities sufficient for stopped-flow analysis lends itself to two applications. The first is that it will allow studies of the impact of perturbations to lipid biosynthesis, trafficking, and turnover on membrane permeability. The rationale for undertaking such studies lies in the fact that little is known about how cells facing detrimental osmotic and chemical gradients, for example, the lumen of the bladder, create a barrier membrane. The biosynthetic and trafficking pathways utilized in such cells are likely to be quite different from most cells that exist in osmotic equilibrium. The oocyte is both pharmacologically and genetically tractable, therefore lending it to experimental approaches that could not easily be used in other cell systems.

**Table 2. Lipid composition of light plasma membranes from Xenopus laevis oocytes as determined by mass spectrometry**

<table>
<thead>
<tr>
<th>Choline Lipids</th>
<th>Ethanolamine Lipids</th>
<th>Anionic Lipids</th>
<th>Other Lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assignment</td>
<td>\textit{m/z}</td>
<td>Mass%</td>
<td>Assignment</td>
</tr>
<tr>
<td>PC</td>
<td>35.8</td>
<td>PE</td>
<td>21.8</td>
</tr>
<tr>
<td>16–0:16:1</td>
<td>732</td>
<td>10.5</td>
<td>18–0:18:1</td>
</tr>
<tr>
<td>16–0:18:2</td>
<td>758</td>
<td>18.1</td>
<td>18–2:20:2</td>
</tr>
<tr>
<td>16–0:18:1</td>
<td>760</td>
<td>33.8</td>
<td>18–2:22:6</td>
</tr>
<tr>
<td>16–0:18:2*</td>
<td>780</td>
<td>19.0</td>
<td>18–1:22:6</td>
</tr>
<tr>
<td>18:2–20:4</td>
<td>806</td>
<td>15.5</td>
<td>ND</td>
</tr>
<tr>
<td>SM</td>
<td>25.8</td>
<td>ND</td>
<td>14:0</td>
</tr>
<tr>
<td>16:0</td>
<td>703</td>
<td>51.6</td>
<td>24:1</td>
</tr>
</tbody>
</table>

Lipid composition excludes cholesterol. PC, phosphatidylcholine; SM, sphingomyelin; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; ND, not determined because signal mass of the initial mass ion was too low to permit other scanning techniques. All ceramide species were detected as Cl\textsuperscript{−} adducts. *Na\textsuperscript{+} adduct.

from most cells that exist in osmotic equilibrium. The oocyte is both pharmacologically and genetically tractable, therefore lending it to experimental approaches that could not easily be used in other cell systems.

**Fig. 4.** Oocyte swelling kinetics for water-injected (inj) and aquaporin-1 (AQP1)-injected oocytes. Oocytes were video imaged as described in METHODS, and then a cross-sectional area was obtained with time and normalized to relative volume. Each curve represents the mean ± SE of 4 oocytes. Arrow indicates point at which oocytes experienced perfusate change from modified Barth’s solution (MBS) to hypotonic (30%) MBS.

**Fig. 5.** AQP1 mediates rapid water efflux kinetics in oocyte membrane vesicles. A: immunoblot of AQP1 in red blood cells (RBC) and in oocyte homogenates (H), postnuclear supernatant (PN), L-membranes (LT), and H-membranes (HV) prepared from nonexpressing (−) and expressing (+) oocytes. Molecular weights (36 and 28) for glycosylated and unglycosylated AQP1, respectively, are shown. B: stopped-flow tracings showing elevated water efflux kinetics for AQP1-expressing L-membranes. R\textsubscript{1} and R\textsubscript{2}, 2 exponential rates fitted to the AQP1 curve. Control membranes were fitted to a single exponential curve. Inset: results from 3 separate sets of oocytes. Values are means ± SE.
cell membranes asymmetrically distribute particular lipids in analysis, however, does not allow us to draw conclusions as to ous clues to explain its low permeability. This compositional the concentration of cholesterol appears to be relatively modest be unusually high in unsaturated acyl chains (22). In addition, 147). Therefore, the lipids in this membrane do not appear to L-cell plasma membrane (UI membranes from a number of different cell types, including 0.6). Compared with rabbit intestinal brush-border membrane, an intrinsic permeability of 8.1 swelling experiments and implies that the membrane itself has an almost complete elimination of channel activity. The almost complete elimination of and urea transporter-containing membranes to demonstrate mediated urea transport. UT-A3 is predominantly expressed on shrinks rates. Characteristically, phloretin inhibited UT-A3-expressing L-membranes. Control membranes were fitted to a single exponential curve. Inset: results from 2 separate sets of oocytes. UT-fast, rapid initial flux rate; phloretin, rate after 0.5 mM phloretin pretreatment for 20–30 min; UT-slow, slow second flux rate. Bars are mean of duplicate experiments with range indicated.

The second application lies in the ability to study the kinetics and regulation of transporters like aquaporins and urea transporters in a system which, while not as compositionally defined as proteoliposomes, offers significant improvements on studies performed in intact oocytes. The results presented here collectively demonstrate that L-membranes are highly enriched for plasma membrane and exhibit permeability coefficients consistent with, and toward the lower end of, those measured for intact oocytes (1, 3, 4, 12, 15, 20, 24, 25). This is an important confirmation of oocyte membrane permeabilities reported on the basis of osmotic swelling experiments and implies that the membrane itself has an intrinsic permeability of $8.1 \times 10^{-4}$ cm/s, a low value that places it in the category of a barrier membrane.

Comparisons with human red cell plasma membrane (2) show the oocyte has a higher concentration of PC (35.8 vs. 29.3), the same amount of SM, lower concentrations of phosphatidylethanolamine (PE; 21.8 vs. 27.6), and phosphatidylserine (5.3 vs. 14.9) but much higher concentrations of PI (6.8 vs. 0.6). Compared with rabbit intestinal brush-border membrane, there is the same amount of PC, less PE, and about the same PI. One very noticeable difference between oocytes and brush-border membrane is a much higher concentration of PC in the oocyte (25.8 vs. 10.3%). Calculation of the unsaturation index (UI), which sums the proportion of each fatty acid multiplied by the number of double bonds, results in a UI for the oocyte plasma membrane of 123. This places it midrange for the membranes from a number of different cell types, including L-cell plasma membrane (UI = 71) and skin fibroblasts (UI = 147). Therefore, the lipids in this membrane do not appear to be unusually high in unsaturated acyl chains (22). In addition, the concentration of cholesterol appears to be relatively modest compared with many cell types. Therefore, there are no obvi- ous clues to explain its low permeability. This compositional analysis, however, does not allow us to draw conclusions as to the distribution of lipids in each leaflet. It is well known that cell membranes asymmetrically distribute particular lipids in the outer and cytoplasmic leaflets (21). It is conceivable that with high concentrations of PC and SM and lower levels of PE, the oocyte maintains an inner leaflet that somewhat resembles the outer leaflet of epithelial cells. We have previously shown that barrier function resides in the outer leaflet (11), so by constructing a membrane with higher than normal concentrations of PC and highly saturated SM in the inner leaflet, permeabilities would be lower. In addition, PC, SM, and cholesterol are thought to form highly ordered domains through hydrogen bonding interactions and for reasons of optimal acyl chain packing (5). The fact that two membrane fractions were isolated with different buoyant densities and that the majority of the plasma membrane was found in the light membranes lends circumstantial support to the presence of membrane microdomains. The presence of all three lipids may explain the low permeability of these membranes to water and urea.

AQP1 and UT-A3 expressed heterologously in oocytes were able to be functionally measured in L-membranes using stopped-flow fluorometry. However, there were two exponen- tial components to the flux curves. That the slow rate was identical to the rate of passive diffusion in control membranes strongly suggests that it is due to passive diffusion rather than some transporter-mediated phenomenon. Fast and slow water fluxes in AQP-expressing L-membranes probably indicate the presence of a population of vesicles without water channels. These may derive from intracellular membrane contaminants that vesiculated and thereby entrapped CF. Contaminating membrane fragments that are not vesiculated do not report any changes in the stopped-flow assay.

UT-A3 expressing membranes also exhibited fast and slow shrinkage rates. Characteristically, phloretin inhibited UT-A3-mediated urea transport. UT-A3 is predominantly expressed on the basolateral membrane of the inner medullary collecting duct and is assumed to mediate urea efflux. Therefore, our data are analogous to and agree with observations made using in vitro perfused inner medullary collecting duct (6). There is clearly sufficient discrimination between control membranes and urea transporter-containing membranes to demonstrate regulation of channel activity. The almost complete elimination of the fast component on treatment of membranes with phloretin demonstrates convincingly that we can study transporter pharmacology, regulation, and kinetics using this system.

In conclusion, we believe that an improved isolation procedure for the plasma membrane of X. laevis oocytes will allow a range of transporters to be studied with much greater precision, control, and reliability than is currently available in the intact oocyte. The removal of unstirred layers from consideration will simplify interpretation and allow a much greater range of experimental manipulations to be performed. The use of vesicles also allows a greater number of transporter substrates and analogs to be tested. This preparation will also be useful for studies of barrier membrane synthesis and may lead to a greater understanding of how coordinate regulation of lipid synthesis and trafficking results in the creation of low-perme- ability membranes.

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