Transalveolar Osmotic and Diffusional Water Permeability in Intact Mouse Lung Measured by a Novel Surface Fluorescence Method

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ABSTRACT A surface fluorescence method was developed to measure transalveolar transport of water, protons, and solutes in intact perfused lungs. Lungs from c57 mice were removed and perfused via the pulmonary artery (~2 ml/min). The airspace was filled via the trachea with physiological saline containing a membrane-impermeant fluorescent indicator (FITC-dextran or aminonapthalene trisulfonic acid, ANTS). Because fluorescence is detected only near the lung surface due to light absorption by lung tissue, the surface fluorescence signal is directly proportional to indicator concentration. Confocal microscopy confirmed that the fluorescence signal arises from fluorophores in alveoli just beneath the pleural surface. Osmotic water permeability ($P_f$) was measured from the time course of intraalveolar FITC-dextran fluorescence in response to changes in perfusate osmolality. Transalveolar $P_f$ was $0.017 \pm 0.001$ cm/s at 23°C, independent of the solute used to induce osmosis (sucrose, NaCl, urea), independent of osmotic gradient size and direction, weakly temperature dependent (Arrhenius activation energy 5.3 kcal/mol) and inhibited by HgCl$_2$. $P_f$ was not affected by cAMP activation but was decreased by 43% in lung exposed to hyperoxia for 5 d. Diffusional water permeability ($P_d$) and $P_f$ were measured in the same lung from intraalveolar ANTS fluorescence, which increased by 1.8-fold upon addition of 50% $\mathrm{D}_2\mathrm{O}$ to the perfusate. $P_d$ was $1.3 \times 10^{-5}$ cm/s at 23°C. Transalveolar proton transport was measured from FITC-dextran fluorescence upon switching perfusate pH between 7.4 and 5.6; alveolar pH half-equilibrated in 1.9 and 1.0 min without and with HCO$_3^-$, respectively. These results indicate high transalveolar water permeability in mouse lung, implicating the involvement of molecular water channels, and establish a quantitative surface fluorescence method to measure water and solute permeabilities in intact lung. Key words: alveolus • osmosis • fluorescent indicator • aquaporin • water channel

INTRODUCTION

There is continuous movement of water, ions, and small solutes between the airspace and blood compartments in the intact lung. Large quantities of fluid move out of the airspaces in the perinatal period, and there is net water movement into the airspaces in the normal adult lung to offset evaporative losses (Boucher, 1994; Olver, 1994). Water and solute transport also play a role in clinically important pathological conditions, including the formation and resolution of alveolar edema (Saumon and Basset, 1993; Matthay et al., 1996) and the correction of alveolar fluid composition after aspiration of acid, salt water, or fresh water. The major barrier between the airspace and blood compartments is the alveolar epithelium, which is in series with the relatively leaky capillary endothelium. In addition, although they comprise much less total surface area (Weibel, 1989), small and large airways also contain a fluid-transporting epithelial cell layer (Folkesson et al., 1996).

The conventional approach to study transalveolar transport of salt and water is to flood the airspaces with fluids of specified osmolality and composition and to assay the composition of serially sampled airspace and/or blood compartment fluids (Basset et al., 1987; Good- man et al., 1987; Effros et al., 1989; Matthay et al., 1996). Various in situ and ex vivo lung preparations have been established for these studies. In the in situ perfused sheep lung, Folkesson et al. (1994b) showed that osmotic equilibration of hyperosmolar (900 mOsm) fluid instilled into the airspaces was rapid and inhibited reversibly by HgCl$_2$. Similar experiments have been performed to measure alveolar fluid composition in response to instillation of acids (Folkesson et al., 1995), fresh water (Acevedo and Robin, 1972), and hypertonic saline (Folkesson et al., 1994a). Significant limitations of this "instill and sample" approach include low time resolution, uncertainties in the sampling of true intraalveolar fluid, the need to use relatively large animals, and the technical difficulty and cost of the studies. The ability to measure transalveolar salt and water transport in mice is particularly important given the recent and anticipated development of transgenic knockout models for lung ion channels (e.g., CFTR) and water transporters.
The purpose of this study was to develop and apply a new approach to measure continuously the composition of intraalveolar fluid in intact mouse lung. Our strategy was to fill the airspace of an isolated perfused mouse lung with physiological saline containing a membrane-impermeant fluorescent indicator. Fluorescent indicators and perfusion strategies were chosen to measure osmotic and diffusional water permeability in the same lung, and to follow the kinetics of intraalveolar pH gradients. For measurement of osmotic water permeability ($P_o$),¹ the airspace was filled with a membrane-impermeant fluorophore at low concentrations in which there was no self-quenching or significant absorption of excitation light or emitted fluorescence (Fig. 1 A). Because of the finite penetration depth of excitation light, only lung tissue near the pleural surface is illuminated so that the surface fluorescence signal is proportional to the airspace fluorophore concentration. In response to an osmotic gradient, water will flow between the airspace and perfusate compartments, resulting in fluorophore concentration or dilution. Fluorophore concentration and thus osmolality is "sampled" by measurement of surface fluorescence. For determination of diffusional water permeability ($P_d$) (Fig. 1 B), the airspace is filled with physiological saline (in $H_2O$) containing the membrane-impermeant fluorophore aminonaphthalene trisulfonic acid (ANTS), whose brightness (quantum yield) depends on solution $H_2O$ vs. $D_2O$ content (Kuwahara and Verkman, 1988). Upon switching the perfusate to an isosmolar solution containing $D_2O$, there is transalveolar exchange of $H_2O$ for $D_2O$ and an increase in surface fluorescence signal; $P_d$ is determined from the time course of surface fluorescence, the relation between ANTS fluorescence vs. solution $D_2O$ content, and alveolar surface-to-volume ratio. The method was applied to characterize in detail the water permeability properties of c57 mouse lung, and to measure the transport of urea and protons. Because high quality quantitative data can be obtained easily in small animals without the need to sample alveolar fluid, the fluorescence method established here should have numerous applications in lung transport physiology.

**METHODS**

**Lung Preparation**

c57 mice (Benton-Kingman, Freemont, CA; 20–25 g) were killed with intraperitoneal pentobarbital (150 mg/kg). The trachea was transected and cannulated in situ with polyethylene PE-50 tubing. The pulmonary artery and left atrium were transected, and the pulmonary artery was cannulated with PE-20 tubing. The cannulae were secured with 3-0 silk surgical thread. The heart and lungs were moved en bloc to a Lucite perfusion chamber for observation by epifluorescence microscopy (Fig. 2). One lung was positioned above a narrow channel (7 mm wide, 10 mm high, 33 mm long) in which HEPES-buffered Ringer's (HBR: 137 mM NaCl, 2.68 mM KCl, 1.25 mM MgSO4, 1.82 mM CaCl2, 5.5 mM glucose, 12 mM HEPES, 1.5% BSA, pH 7.4, 300 mOsm) was continuously perfused (10–20 ml/min) between the external pleural surface and the coverglass. The pulmonary artery was gravity perfused at constant pressure (25–35 cm $H_2O$) using a multi-solution reservoir and 4-way flow valve (vascular resistance 20–30 cm $H_2O$/mm per min). The pulmonary artery was initially perfused with HBR for 5 min to remove all blood and to set reservoir height for the desired perfusion rate (generally 1–2.5 ml/min). In some experiments, a membrane-impermeant fluorophore (FITC-dextran) was added to the perfusate solution. The temperature of the pulmonary artery and pleural surface perfusates were controlled by circulating water around the perfusion inflow tubing; effluent temperature was measured by a thermistor positioned near the outflow. The airspace was filled with 0.5–0.8 ml

¹Abbreviations used in this paper: ANTS, aminonaphthalene trisulfonic acid; $P_d$, diffusional water permeability; $P_o$, osmotic water permeability.
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**FIGURE 2.** Instrumentation for measurement of pleural surface fluorescence in the ex vivo perfused mouse lung. Lungs and heart were positioned en bloc in a perfusion chamber on the stage of an inverted epifluorescence microscope. The trachea was cannulated for instillation of saline containing a fluorescent indicator. The pulmonary circulation was perfused via the pulmonary artery and the pleural surface was washed continuously with saline. Perfusate and wash effluent was withdrawn from the chamber by suction.

Fluorescence was recorded from a 3-5 mm diameter spot on the lung surface. See text for details.

of HBR containing FITC-dextran (70 KDa, 0.5-5 mg/ml; Sigma Chemical Co., St. Louis, MO) or aminonaphthalene trisulfonic acid (ANTS, 5 mM, Molecular Probes Inc., Eugene, OR). Fluorescence measurements were carried out in a dark room. In some experiments, mice were exposed to 100% oxygen for 5 d to produce endothelial and mild epithelial lung damage (Frank et al., 1978; Grapo, 1986).

### Pleural Surface Fluorescence Microscopy

The fluorescence intensity from a 3-5 mm diameter spot on the lung pleural surface was monitored using an inverted epifluorescence microscope (Nikon Diaphot) (Fig. 2). The spot was illuminated using a stabilized tungsten-halogen lamp (15 V, 5 amps) in series with a neutral density filter (optical density 1.0), interference filter, and dichroic mirror. A 10X dry objective (Leitz, numerical aperture 0.25) was used for all measurements. Emitted fluorescence was filtered by a cut-on and detected by a photomultiplier (R928S; Hamamatsu, Middlesex, NJ). Filter wavelength specifications were (interference, dichroic, cut-on): FITC~dextran (480 ± 5 nm, 510 nm, >515 nm); ANTS (380 ± 10 nm, 430 nm, >515 nm). The photomultiplier signal was amplified by: faro = -0.11(F/Fo) 2 + 0.91(F/Fo) - 0.79 (Folkesson et al., 1996). Since dfD2O/dt = [dfD2O/d(F/Fo)] [d(F/Fo)/d(Raw)] Pd can be determined from dfD2O/dt at zero time, just after addition of 50% D2O (ΔD2O = 0.5) to the perfusate, Pd = [df(F/Fo)/dR]1 - r [dfD2O/d(F/Fo)]1 - r / [0.5/S(Vo)].

### Diffusional Water Permeability

The airspace was filled with HBR (in 100% H2O) containing ANTS, a membrane-impermeant fluorophore whose quantum yield is insensitive to ionic strength and pH, but increases 3.2-fold when H2O is replaced by D2O (Kuwahara and Verkman, 1988). The pulmonary artery was initially perfused with HBR: in 100% H2O. The perfusate was then switched to isosmolar HBR prepared in 50% H2O-50% D2O. Diffusional exchange of D2O with H2O resulted in an increase in intraalveolar D2O content and thus increased ANTS fluorescence.

Diffusional water permeability is defined by: dfD2O/dt = Pd(S/Vo) ΔD2O, where fD2O (0 to 1) is the fractional content of D2O, and Pd (cm/s) is the diffusional water permeability coefficient. As determined by fluorimetry, fD2O is related to relative ANTS fluorescence, F/Fo by: fD2O = -0.11(F/Fo) 2 + 0.91(F/Fo) - 0.79 (Folkesson et al., 1996). Since dfD2O/dt = [dfD2O/d(F/Fo)] [d(F/Fo)/dR] Pd can be determined from dfD2O/dt at zero time, just after addition of 50% D2O (ΔD2O = 0.5) to the perfusate, Pd = [df(F/Fo)/dR] 1 - r [dfD2O/d(F/Fo)] 1 - r / [0.5/S(Vo)].

### Osmotic Water Permeability

The airspace was filled with HBR (300 mOsm, measured by osmometer; Advanced Instruments, Needham Heights, MA) containing either FITC-dextran or ANTS, and the pulmonary artery was perfused with HBR for at least 5 min. An airspace-to-perfusate osmotic gradient was generated by switching the perfusate to distilled water (hypoosmolar). In some experiments NaCl or urea were used in place of sucrose. The time course of lung surface fluorescence was monitored continuously. A series of perfusate fluid exchanges and transport measurements could be done for >1 h without change in vascular resistance or water permeability.

Osmotically induced volume flux, Jv (cm3/s), is defined by: Jv = dV/dt = Pw S ΔCw, where Pw (cm/s) is the osmotic water permeability coefficient, S (cm2) is surface area, ΔCw (18 cm2/mol) is the partial molar volume of water, and ΔC is the difference in osmolality between perfusate and airspace fluids. Because the surface fluorescence signal, F(t), is directly proportional to intraalveolar fluorophore concentration (see RESULTS) and thus to intravascular fluid osmolality, it follows that F(t) = F(t)0 when F(t)0 is initial fluorescence and S is initial airspace fluid volume. Differentiating, dV/dt = - [F(t)V(t)]/F(t0) dF(t0)/dt, and utilizing the Jv definition above: dF(t)/dt = - (F(t)/F(t0))PwS/(S/Vo) ΔC. Evaluating dF(t)/dt at zero time (where F = F0),

Pw = [d(F/F0)/dt]o / [(S/Vo) v ΔC],

where [d(F/F0)/dt]o is computed from the initial slope of a biexponential fit to the measured fluorescence time course, and surface-to-volume ratio, S/Vo (cm-1), is determined from serial confocal micrographs of surface alveoli (see below). Eq. 1 assumes that osmolality is constant throughout the airspace compartment and that alveolar surface area constitutes the major contribution to total airspace surface area. Eq. 1 also assumes that osmotically active solutes have a unity reflection coefficient.

In experiments in which higher airspace fluorophore concentrations were used, F(t) is not directly proportional to fluorophore concentration because of inner filter effects (absorbance of light by fluorophores); the right side of Eq. 1 is then multiplied by (Cp/Ca - 1)/[Fmax/F - 1], where Cp/Ca is the ratio of perfusate to initial airspace fluid osmolality and Fmax is final fluorescence.

### Results

**FIGURE 2.** Instrumentation for measurement of pleural surface fluorescence in the ex vivo perfused mouse lung. Lungs and heart were positioned en bloc in a perfusion chamber on the stage of an inverted epifluorescence microscope. The trachea was cannulated for instillation of saline containing a fluorescent indicator. The pulmonary circulation was perfused via the pulmonary artery and the pleural surface was washed continuously with saline. Perfusate and wash effluent was withdrawn from the chamber by suction. Fluorescence was recorded from a 3-5 mm diameter spot on the lung surface. See text for details.

- **Figure 2** shows the instrumentation for measuring pleural surface fluorescence. The lungs and heart were positioned in a perfusion chamber on the stage of an inverted epifluorescence microscope. The trachea was cannulated for instillation of saline containing a fluorescent indicator. The pulmonary circulation was perfused via the pulmonary artery, and the pleural surface was washed continuously with saline. Perfusate and wash effluent were withdrawn from the chamber by suction. Fluorescence was recorded from a 3-5 mm diameter spot on the lung surface.

- **Figure 3** illustrates the measurement of intravascular and alveolar H2O content. The airspace was filled with HBR (300 mOsm) and then perfused with HBR containing FITC-dextran or ANTS. The pulmonary artery was initially perfused with HBR: in 100% H2O. The perfusate was switched to isosmolar HBR prepared in 50% H2O-50% D2O. Diffusional exchange of D2O with H2O resulted in an increase in intraalveolar D2O content and thus increased ANTS fluorescence.

- **Figure 4** demonstrates the measurement of diffusional water permeability. The airspace was filled with HBR (in 100% H2O) containing ANTS, a membrane-impermeant fluorophore whose quantum yield is insensitive to ionic strength and pH, but increases 3.2-fold when H2O is replaced by D2O. The pulmonary artery was initially perfused with HBR: in 100% H2O. The perfusate was then switched to isosmolar HBR prepared in 50% H2O-50% D2O. Diffusional exchange of D2O with H2O resulted in an increase in intraalveolar D2O content and thus increased ANTS fluorescence.

- **Figure 5** shows the measurement of osmotic water permeability. The airspace was filled with HBR (300 mOsm, measured by osmometer; Advanced Instruments, Needham Heights, MA) containing either FITC-dextran or ANTS, and the pulmonary artery was perfused with HBR for at least 5 min. An airspace-to-perfusate osmotic gradient was generated by switching the perfusate to distilled water (hypoosmolar). In some experiments NaCl or urea were used in place of sucrose. The time course of lung surface fluorescence was monitored continuously. A series of perfusate fluid exchanges and transport measurements could be done for >1 h without change in vascular resistance or water permeability.
above by switching the perfusate to an isosmolar solution containing 50% D\textsubscript{2}O.

Proton Transport Measurements

The airspace was filled with HBR containing 1 mg/ml FITC-dextran (pH 6.4) for continuous measurement of intraalveolar pH. The perfusate consisted of HBR (titrated to pH 7.4 or 5.5) or HBR in which 25 mM Cl\textsuperscript{-} was replaced with HCO\textsubscript{3}\. Surface fluorescence intensity was converted to airspace fluid pH from an in vitro FITC-dextran titration.

Confocal Microscopy

Confocal microscopy was carried out to visualize the fluorophore distribution and to determine alveolar geometry. The airspace was filled with HBR containing FITC-dextran and the pulmonary artery was perfused as described above. The pleural surface was viewed using a Leitz upright epifluorescence microscope with co-axial-confocal attachment (Technical Instruments, San Francisco, CA). A coverslip (thickness 0.17 mm) was positioned over the lung surface by a rigid support for visualization of fluorescently stained alveoli with a 60× Plan-Apo objective (Nikon, oil immersion, numerical aperture 1.4). Images (512 × 512 pixels, 14-bit resolution) were acquired with a cooled CCD camera using PMIS software (Photometrics, Tucson, AZ) (Zen et al., 1992). The instrument z-resolution for rejection of out-of-focus fluorescence was ~1 μm. Alveolar surface-to-volume ratio was computed from serial images acquired 4.5 μm apart using a three-dimensional image reconstruction procedure reported previously (Farinas et al., 1995).

RESULTS

Experiments were first carried out to verify the alveolar origin of the surface fluorescence signal and to show that the airspace fluorophore remains confined to the airspace compartment. Fig. 3 A shows a low magnification confocal micrograph of the lung surface after filling the airspace with physiological saline (HBR) containing FITC-dextran. Individual alveoli are seen with nonfluorescent septa separating alveoli, as well as a nonfluorescent blood vessel. At higher magnification, the geometry of individual alveoli are clearly visualized (Fig. 3 B). After changing perfusate osmolality to 600 mOsm or 150 mOsm, there was no obvious change in alveolar geometry as seen in representative confocal micrographs in Fig. 3, C and D. To examine quantitatively the effect of perfusate osmolality on alveolar geometry, the three-dimensional alveolar shape was reconstructed from serial confocal micrographs acquired at a series of z-positions (Fig. 3 E). The apparent alveolar size increased to a mean diameter of ~40 μm as the z-focus traversed individual alveoli. Well-resolved images could be obtained to ~25 μm beneath the lung surface because of light scattering by lung tissue. By image reconstruction (Farinas et al., 1995), alveolar surface-to-volume (S/V) ratio was computed to be 1,626 ± 107 cm\textsuperscript{-1} with the 300 mOsm perfusate, not significantly different from that of 1,533 ± 219 cm\textsuperscript{-1} with the 600 mOsm perfusate. These values are similar to the S/V of 1,500 cm\textsuperscript{-1} predicted for spherical alveoli of 40 μm diameter, as reported in morphological studies of mouse lung (Milsom, 1989). The S/V value is required for computation of absolute P\textsubscript{f} and P\textsubscript{a} (Eqs. 1 and 2). Measurements of perfusate effluent fluorescence indicated that <2% of airspace fluorophore appeared in the perfusate over 1 h. These studies indicate that the surface fluorescence signal arises from fluorophores confined to the airspace compartment.

Fig. 4 A shows the time course of lung surface fluorescence in response to changes in perfusate osmolality. In response to a sudden increase in perfusate osmolality to 600 mOsm, there was a prompt rise in fluorescence without a lag phase; final fluorescence was approximately twice the initial fluorescence. Perfusion with the original isosmolar solution returned the signal to baseline (without photobleaching). Final fluorescence decreased approximately twofold with the 150 mOsm perfusate. The quantitative agreement of the fluorescence signal change with perfusate osmolality indicates that surface fluorescence is a valid indicator of intraalveolar fluorophore concentration and that the fluorophore is confined to the airspace compartment (and not in the interstitium or vascular compartment). To estimate the effective penetration depth of the excitation light, the same experiment was performed at a higher concentration of FITC-dextran (5 mg/ml), where...
significant absorption of excitation light (inner filter effect) should occur (Fig. 4 B). Although the curve shape was similar to that for the low FITC-dextran concentration (Fig. 4 A), the amplitude was much less than that predicted for a dilute fluorophore. Perfusion with the 600 mOsm solution gave a relative amplitude of 1.35 (2.0 predicted for dilute fluorophore); with the 150 mOsm perfusate, the amplitude was 0.65 (0.5 predicted). The attenuated signal amplitude results from a time-dependent change in lung light absorbance (sum of lung tissue and FITC absorbances) due to concentration or dilution of the intraalveolar FITC-dextran. Using these results and the measured optical density of FITC-dextran at 480 nm (0.18 for 5 mg/ml FITC-dextran, 1 mm pathlength), the effective light penetration depth is estimated to be ~300 μm for mouse lung in the absence of airspace fluorophore.

To compute Pr from the time course of surface fluorescence, the exchange of perfusion solutions must be fast compared to the kinetics of surface fluorescence change. Fig. 4 C shows the time course of lung surface fluorescence in response to addition and removal of FITC-dextran to the perfusate, where the airspace was filled with HEPES-buffer Ringer's (HBR) not containing fluorophore. The appearance and disappearance of fluorophore in the vascular space was very rapid (half-times of 12 s, 1 ml/min perfusate flow; 8.5 s, 1.25 ml/min; 4.5 s, 2 ml/min) and much faster than the half-times for osmotic equilibration in Fig. 4. A and B (half-times ~40 s). For a 600 mOsm perfusate, measured Pr values at 25°C were independent of perfusate flow rate (Pr = 0.016 cm/s, 1.36 ml/min; 0.015 cm/s, 1.67 ml/min; 0.017 cm/s, 2 ml/min), supporting the conclusion that measured Pr depends on the characteristics of the airspace-to-capillary barrier and is not limited by vascular flow rate. Further support for the adequacy of vascular perfusion was the lack of effect of papaverine (a vasodilator) on osmotically induced water flow (see Fig. 5 E). In addition, Pr was not dependent upon airspace pressure (0–5 cm H2O) or the location of the illuminated spot on the lung surface (data not shown).

Osmotic water transport was further characterized in Fig. 5. Fig. 5 A shows that Pr for water movement between the airspace and perfusate compartments did not depend significantly on osmotic gradient size and direction. Average Pr was 0.017 ± 0.002 cm/s (SD, 23°C), a high value compared to most other biological membranes (see DISCUSSION), suggesting the involvement of molecular water channels. Fig. 5 B shows that osmotic water transport induced by equal osmotic gradients of sucrose, NaCl, and urea was similar (Pr = 0.017 ± 0.002 cm/s, sucrose; 0.018 ± 0.003, NaCl; 0.017 ± 0.001, urea), indicating that the reflection coefficients for NaCl and urea are near unity. In response to addition of 300 mM urea to the perfusate (final osmolality 600 mOsm), fluorescence increased promptly and remained elevated for >20 min (Fig. 5 B, dashed curve). This finding indicates very low permeability for urea movement between the airspace and capillary compartments. Fig. 5 C shows that Pr was weakly temperature dependent. An Arrhenius plot of ln Pr vs. reciprocal absolute temperature gave a slope (representing the activation energy, E_a) of 5.3 ± 1 kcal/mol. The low activation energy further supports the involvement of water channels (Verkman, 1989). Fig. 5 D shows that perfusate HgCl₂, a known inhibitor of water channels AQP-1 and AQP-5 (Preston et al., 1992; Van Hoek and Verkman, 1992; Raina et al., 1995), partially inhibits osmotic water movement in intact lung. Averaged Pr values are summarized in Fig. 5 E. Pr was decreased significantly by HgCl₂, but not by other known effectors of

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**Figure 4.** Measurement of osmotic water movement between airspace and capillary compartments in mouse lung. (A and B) Pleural surface fluorescence was recorded continuously in response to indicated changes in perfusate osmolality. The airspace was filled with HBR containing 0.5 mg/ml (A) or 5 mg/ml (B) FITC-dextran. (C) To measure perfusate exchange time, the airspace was filled with nonfluorescent HBR and the pulmonary artery was perfused at indicated flow rates with HBR containing 0 (−F) or 0.5 mM (+F) FITC-dextran.

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**Figure 5.** Osmotic water transport was further characterized in Fig. 5. Fig. 5 A shows that Pr for water movement between the airspace and perfusate compartments did not depend significantly on osmotic gradient size and direction. Average Pr was 0.017 ± 0.002 cm/s (SD, 23°C), a high value compared to most other biological membranes (see DISCUSSION), suggesting the involvement of molecular water channels. Fig. 5 B shows that osmotic water transport induced by equal osmotic gradients of sucrose, NaCl, and urea was similar (Pr = 0.017 ± 0.002 cm/s, sucrose; 0.018 ± 0.003, NaCl; 0.017 ± 0.001, urea), indicating that the reflection coefficients for NaCl and urea are near unity. In response to addition of 300 mM urea to the perfusate (final osmolality 600 mOsm), fluorescence increased promptly and remained elevated for >20 min (Fig. 5 B, dashed curve). This finding indicates very low permeability for urea movement between the airspace and capillary compartments. Fig. 5 C shows that Pr was weakly temperature dependent. An Arrhenius plot of ln Pr vs. reciprocal absolute temperature gave a slope (representing the activation energy, E_a) of 5.3 ± 1 kcal/mol. The low activation energy further supports the involvement of water channels (Verkman, 1989). Fig. 5 D shows that perfusate HgCl₂, a known inhibitor of water channels AQP-1 and AQP-5 (Preston et al., 1992; Van Hoek and Verkman, 1992; Raina et al., 1995), partially inhibits osmotic water movement in intact lung. Averaged Pr values are summarized in Fig. 5 E. Pr was decreased significantly by HgCl₂, but not by other known effectors of

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**Figure 6.** Osmotic water transport was further characterized in Fig. 5. Fig. 5 A shows that Pr for water movement between the airspace and perfusate compartments did not depend significantly on osmotic gradient size and direction. Average Pr was 0.017 ± 0.002 cm/s (SD, 23°C), a high value compared to most other biological membranes (see DISCUSSION), suggesting the involvement of molecular water channels. Fig. 5 B shows that osmotic water transport induced by equal osmotic gradients of sucrose, NaCl, and urea was similar (Pr = 0.017 ± 0.002 cm/s, sucrose; 0.018 ± 0.003, NaCl; 0.017 ± 0.001, urea), indicating that the reflection coefficients for NaCl and urea are near unity. In response to addition of 300 mM urea to the perfusate (final osmolality 600 mOsm), fluorescence increased promptly and remained elevated for >20 min (Fig. 5 B, dashed curve). This finding indicates very low permeability for urea movement between the airspace and capillary compartments. Fig. 5 C shows that Pr was weakly temperature dependent. An Arrhenius plot of ln Pr vs. reciprocal absolute temperature gave a slope (representing the activation energy, E_a) of 5.3 ± 1 kcal/mol. The low activation energy further supports the involvement of water channels (Verkman, 1989). Fig. 5 D shows that perfusate HgCl₂, a known inhibitor of water channels AQP-1 and AQP-5 (Preston et al., 1992; Van Hoek and Verkman, 1992; Raina et al., 1995), partially inhibits osmotic water movement in intact lung. Averaged Pr values are summarized in Fig. 5 E. Pr was decreased significantly by HgCl₂, but not by other known effectors of
FIGURE 5. Properties of osmotic water permeability (P\textsubscript{t}) in mouse lung. (A) P\textsubscript{t} (mean ± SD, n = 4–5, 23°C) for indicated initial and final perfusate osmolalities. (B) Osmotic water movement induced by gradients of sucrose, NaCl, and urea. Indicated 600 mOsm solutions consisted of HBR containing 300 mOsm of added solute. (C, left) Representative surface fluorescence data for measurements performed at indicated temperatures. (right) Arrhenius plot for temperature dependence of P\textsubscript{t} with slope (activation energy) of 5.3 kcal/mol (1 cal = 4.184 J). (D) Inhibition of osmotic water permeability by 0.1 mM HgCl\textsubscript{2} (added to perfusate, 23°C). (E) Summary of P\textsubscript{t} values (SD, n = 4–10) measured in the presence of perfusate HgCl\textsubscript{2} (0.1 mM), isoproterenol (0.1 mM), forskolin (20 μM), or papaverine (0.1 mg/ml).

To define further the properties of water transport in mouse lung, diffusional water permeability was measured. Fig. 6 A shows the time course of lung surface ANTS fluorescence in response to addition of 50% D\textsubscript{2}O to the perfusate. There was a very rapid increase in fluorescence without a lag phase; fluorescence returned to the baseline value after perfusion with the original isosmolar H\textsubscript{2}O buffer. The rate of fluorescence change increased with higher perfusion flow (from 1.7 to 2.0 ml/min), but did not change with a further increase in flow to 2.3 ml/min. The apparent half-time for the change in fluorescence at 1.8 ml/min perfusion (~33 s) was less than that in the P\textsubscript{f} measurement (Fig. 4 A), yet slower than the perfusion exchange time (Fig. 4 C). Calculated P\textsubscript{d} values at 23°C were 1.0 × 10\textsuperscript{-5} cm/s (1.7 ml/min perfusion flow), 1.5 × 10\textsuperscript{-5} cm/s (2.0 ml/min) and 1.6 × 10\textsuperscript{-5} cm/s (2.3 ml/min). These low P\textsubscript{d} values indicate that diffusional water permeability is unstirred layer limited (see Discussion). Fig. 6 B shows a measurement of P\textsubscript{f} and P\textsubscript{d} in the same lung in which the air-space was filled with physiological saline containing ANTS. In response to perfusion with hyperosmolar H\textsubscript{2}O-containing buffer, ANTS fluorescence increased as a consequence of osmotic water movement. The fluorescence returned to the original level after perfusion.

l lung transport processes which act acutely, including isoproterenol and forskolin, or by the vasodilator papaverine.
with the isosmolar H$_2$O solution. Subsequent perfusion with the isosmolar 50% D$_2$O solution gave increased fluorescence as a consequence of diffusional water movement. Averaged permeability values for four sets of measurements at 23°C were $P_t = 0.012 \pm 0.002$ cm/s and $P_d = (0.7 \pm 0.2) \times 10^{-5}$ cm/s.

Because $P_d$ is unstirred layer limited and thus an indicator of vascular perfusion efficiency, measurement of $P_t$ and $P_d$ in the same lung is useful to evaluate the effect of lung injury on water transport. Fig. 6 C shows water permeability measurements on lungs from mice exposed to 100% oxygen for 5 d, which produces significant endothelial cell injury and some interstitial changes and epithelial cell damage (Frank et al., 1978; Crapo, 1986). In four mice (a fifth mouse was ill and not studied), averaged $P_t$ decreased significantly by 43% (Fig. 6, inset), whereas $P_d$ was unchanged. These results indicated that hyperoxia-induced lung injury is associated with decreased water permeability.

To demonstrate one other application of the surface fluorescence method, proton transport between the airspace and capillary compartments was measured. Fig. 7 shows the time course of intraalveolar pH, measured by FITC-fluorescence, in response to switching perfusate pH between 7.4 and 5.6 in the absence and presence of HCO$_3^-$.

The half-times for pH equilibration were ~1.9 min, which taken together with solution buffer capacity, gave an apparent proton permeability coefficient ($P_{H^+}$), defined as in Verkman, (1987) of 0.06 cm/s. Alveolar pH returned to 7.4 promptly upon perfusion with the original solution, a finding with implications for the pathophysiology of acute acid aspiration (Folkesson et al., 1995). Upon addition of HCO$_3^-$ to the perfusate, there was a transient decrease in alveolar pH corresponding to rapid perfusate-to-airspace movement of CO$_2$, followed by slower HCO$_3^-$ movement and HCO$_3^-$/CO$_2$ equilibration. Subsequent perfusion with acidic and neutral buffers have pH equilibration half-times of ~1 min, suggesting the participation of a HCO$_3^-$-dependent transporter.

**DISCUSSION**

The purpose of this study was to establish a surface fluorescence method to quantify water and solute permeability in intact lungs in order to characterize transalveolar fluid composition. Intraalveolar osmolality and thus osmotic water permeability was inferred from indicator fluorescence intensity, exploiting the change in indicator concentration resulting from transalveolar water movement. Diffusional water permeability was measured using a fluorescent indicator having an H$_2$O-D$_2$O sensitive quantum yield. Transport of protons was measured using an intraalveolar pH indicator. Because nearly all of the fluorescence signal from the lung pleural surface originated from indicator in the intraalveolar compartment, the surface fluorescence signal provided an instantaneous and quantitative measure of intraalveolar fluid composition. Compared to previous approaches to measure transport between the alveolar and capillary
compartments, the surface fluorescence approach is technically simple and sensitive, and provides continuous information about intraalveolar fluid composition without the need to obtain samples from the distal airspace. In addition, unlike airspace fluid sampling methods, the surface fluorescence approach permits measurement of rapid transport processes in small animals.

The high permeability coefficient \( (P_l = 0.017 \text{ cm/s at 23°C}) \) for osmotically driven water movement between the airspace and capillary compartments in mouse lung suggests the involvement of molecular water channels. For comparison, \( P_l \) is 0.0005-0.003 in the relatively water impermeable epithelium of thick ascending limb of Henle and the unstimulated kidney collecting duct (for review, see Finkelstein, 1989; Verkman, 1989). Examples of highly water permeable epithelia include the kidney proximal tubule \( (P_l = 0.1-0.5 \text{ cm/s}) \) and the vasopressin-stimulated collecting duct \( (0.02 \text{ cm/s}) \). In a previous study in sheep lung, a lower limit for airspace-to-capillary \( P_l \) was estimated to be 0.01 cm/s at 20°C (Folkesson et al., 1994b), although this value had considerable uncertainty because the intratracheal installation time was comparable to the half-time for osmotic equilibration and because of difficulties in sampling airspace fluid. In the mouse lung here, osmotic water permeability was weakly temperature dependent and inhibited by HgCl₂, providing strong support for transalveolar water movement through molecular water channels.

The diffusional water permeability coefficient \( (P_d) \) in mouse lung was measured to be \( 1.3 \times 10^{-5} \) at 23°C, giving an apparent \( P_l/P_d \) ratio of 1,300. In general, a \( P_l/P_d \) ratio of greater than unity is consistent with water passage through a pore-containing pathway (Finkelstein, 1989); however, an alternative explanation is that measured \( P_d \) underestimates true cell \( P_d \) because of the presence of unstirred layers. The unstirred layer can comprise barriers for water diffusion within cells, in the interstitium, and/or within the alveolus. Based on measurements in other water permeable epithelia such as the kidney proximal tubule and the vasopressin-stimulated collecting duct and amphibian urinary bladder (Levine et al., 1984; Verkman, 1989), a significant unstirred layer is predicted for transalveolar \( P_d \), even if the interstitium and endothelial cell layer posed little resistance to water diffusion. Although the precise location of the unstirred layer barrier for \( P_d \) cannot be identified in this study, the high \( P_l/P_d \) compared with other tissues \( (P_l/P_d = 100, \text{proximal tubule}; 10-15, \text{collecting duct}; 17, \text{toad bladder}) \) suggests multiple barriers to water diffusion between the airspace and capillary compartments. It is noted that transepithelial osmotic water transport is relatively resistant to unstirred layer effects because of bulk water movement (Barry and Diamond, 1984). Unstirred layers for \( P_l \) arise from solute polarization near membrane surfaces due to convection-diffusion, rather than to simple diffusive barriers in the case of diffusional transport of water or solutes. The measured osmotic water permeability here is probably not much affected by unstirred layers based on the high \( P_l \), low activation energy \( (E_a) \), and mercurial inhibition as well as the independence of \( P_l \) on osmotic gradient size and direction. Therefore, as found in several other epithelia, unstirred layers have a substantial effect on the determination of diffusional, but not osmotic water permeability.

A physiological role for lung water channels is suggested but not proven by the high airspace-to-capillary water permeability found here (for review of water channels, see Verkman et al., 1996; Agre and Nielsen, 1995). The AQP-1 (CHIP28) water channel is expressed primarily on alveolar capillary endothelia (Nielsen et al., 1993; Folkesson et al., 1994a; Hasegawa et al., 1994a) and to a lesser extent on type II alveolar epithelial cells (Folkesson et al., 1994b), which comprise a minority of alveolar surface area. The alveolar capillary endothelium probably poses a minor barrier to water and solute transport (Wangensteen, 1994). Furthermore, the normal phenotype of subjects lacking AQP-1 (Preston et al., 1994) suggests that AQP-1 is not re-

![Figure 7. Proton transport measured in mouse lung by pleural surface fluorescence. The airspace was filled with HBR containing 1 mM FITC-dextran and the pulmonary artery was perfused with HBR at indicated pH or a HCO₃⁻-containing solution (see METHODS).](image-url)
quired physiologically in lung. The mercurial-insensitive water channel (MIWC, AQP-4) (Hasegawa et al., 1994b; Yang et al., 1995, 1996) is primarily expressed at the basolateral membrane of airway epithelia (Frigeri et al., 1995), and probably facilitates trans-airway but not transalveolar water transport (Folkesson et al., 1996). A third water channel which is mercurial-sensitive (AQP-5) (Raina et al., 1995) is strongly expressed in lung and appears to be localized primarily to type I alveolar epithelia (unpublished results). The high transalveolar $P_f$ measured here may thus be accounted for by AQP-5. In recent studies, expression of the three water channels in lung function will require studies in transgenic knock-out mice or the development of nontoxic and selective water channel inhibitors.

There are several technical concerns in the application of the surface fluorescence method to measure airspace-capillary permeabilities. Potential serial permeability barriers exist between the capillary and airspace compartments, including capillary endothelial cells, the interstitium, alveolar epithelial cells, and aqueous-space unstirred layers. In general, the alveolar epithelium is believed to represent the major barrier to transport of small solutes (Wangensteen, 1994). The prompt fluorescence signal increase found here upon a change in perfusate fluid composition (Fig. 3 A) is consistent with the alveolar epithelium comprising the primary permeability barrier; a lag phase might be observed if the interstitium or endothelium pose a significant barrier to osmosis. In any epithelium, unstirred layers might influence apparent transport rates for solutes having high permeabilities, as was found here for diffusional water transport. Another concern in the interpretation of results is heterogeneity in alveolar size and transport properties. The experiments were carried out using a photomultiplier to integrate the signal arising from many individual alveoli and thus gave averaged permeability values. Alternatively, image analysis can be used to quantify permeability properties of many individual alveoli at the same time or to visualize a large area of lung surface to study regional heterogeneity. In addition, although no data are available, possible differences in transport properties of surface vs. deep alveoli should be considered. Finally, it is important to recognize that changes in the fluorescence of an intralveolar indicator may arise both from changes in indicator concentration, resulting from osmotic water movement, as well as changes in indicator quantum yield resulting from specific indicator-solute interactions.

Notwithstanding these concerns, the surface fluorescence approach described here should have numerous applications in the quantitative measurement of water, solute, and ion transport in ex vivo and in vivo lungs. Transport of ions such as $\text{Cl}^-$, $\text{Na}^+$, and $\text{K}^+$ are measurable using suitable membrane-impermeant fluorescent indicators in the airspace (Verkman, 1995). Transport studies can be performed in lungs from small or large animals, provided that the airspace can be filled with saline containing appropriate fluorescent indicators. Our method should be particularly useful for transport measurements in control vs. transgenic mice and for studies of spontaneous fluid transport in intact lung under physiological conditions.

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