Transcellular water transport in lung alveolar epithelium through mercury-sensitive water channels

HANS G. FOLKESSON, MICHAEL A. MATTHAY*, HAJIME HASEGAWA, FARRAH KHERADMAND, AND A. S. VERKMAN

Department of Medicine and Physiology, Cardiovascular Research Institute, University of California, San Francisco, CA 94143-0130

Communicated by John A. Clements, February 9, 1994 (received for review September 28, 1993)

ABSTRACT The movement of water between the air space and capillary compartments is important for the maintenance of air space hydration during respiration and for reabsorption of excess alveolar fluid. We have obtained immunocytochemical and functional evidence that plasma-membrane water channels are responsible for water transport in the intact lung. Northern and quantitative immunoblot analysis showed high expression of CHIP28 (channel-forming integral membrane protein of 28 kDa) water channels in rat lung; immunocytochemistry showed CHIP28 localization to epithelial cell plasma membranes. Stopped-flow light scattering measurements of osmotic water permeability ($P_f$) in freshly isolated rat alveolar type II epithelial cells indicated a high $P_f$ of 0.015 ± 0.002 cm/s (10°C) that was weakly temperature-dependent (activation energy, 4 kcal/mol) and reversibly inhibited by 78 ± 4% by 0.5 mM HgCl$_2$. An in situ-perfused sheep lung model was used to determine the route for water movement in intact lung. Blood-to-air-space water transport was measured by sampling air space fluid after instillation into distal air spaces of hyperosmolar saline (900 mOsm) containing radiolabeled albumin and $[^{14}C]$mannitol. In seven sets of experiments, air space osmolality and radiolabeled albumin equilibrated with a $t_{1/2}$ of 0.85 ± 0.1 min. In the contralateral lung perfused with 0.5 mM HgCl$_2$, $t_{1/2}$ increased to 2.7 ± 0.4 min; the inhibitory effect of HgCl$_2$ was fully reversed by 5 mM 2-mercaptoethanol. These results provide direct evidence for transcellular movement of water across the alveolar epithelium in intact lung through mercury-sensitive water channels.

Substantial quantities of water move into the air spaces to offset insensible water losses associated with respiration and move out of the air spaces in the resolution of alveolar edema. Large quantities of water also move across air space epithelia in the fetal and newborn lung. In general, transepithelial water movement is driven by osmotic gradients produced by active ion transport (1-5). Both in vivo and in vitro studies have recently demonstrated the central role of active Na$^+$ transport across the alveolar epithelium in driving the reabsorption of excess fluid from distal air spaces of the lung (2, 4, 6-8). Although a considerable body of information has been established on the mechanisms of ion transport in lung, the pathways and mechanisms for the transport of water across lung epithelia have not been identified.

Recently, an abundant water transport protein (the channel-forming integral membrane protein of 28 kDa (CHIP28)) has been identified in erythrocytes (9, 10) and in several transporting epithelia, including kidney proximal tubule and thin descending limb of Henle (11, 12), intestinal crypt, choroid plexus, and ciliary body (13, 14). CHIP28 is a glycoprotein that transports water selectively when reconstituted into proteoliposomes (15, 16) and when expressed in Chinese hamster ovary (CHO) cells (17) and Xenopus oocytes (18, 19). CHIP28 water transport is inhibited by HgCl$_2$ at Cys-189 (20). CHIP28 probably contains independently functional monomeric units (21) that form tetramers in cell membranes (22); each monomer contains four membrane-spanning $\alpha$-helices at the endoplasmic reticulum membrane (23, 24). In situ hybridization indicated that mRNA encoding CHIP28 is present in alveolar epithelium of rat lungs (13). Recently, several cDNAs homologous to CHIP28 have been cloned (23, 25), including a mercurial-insensitive water channel (MIWC) from rat lung (27). The role of these proteins in lung water movement has not been established, nor is it known whether the primary route for trans-alveolar water movement is transcellular or paracellular.

The purpose of this study was to determine the role of mercury-sensitive water channels in trans-alveolar water transport in lung. Northern and immunoblot analyses indicated strong CHIP28 expression in lung, and immunostaining localized CHIP28 to alveolar and airway epithelial cells. Measurements of osmotic water permeability were done to determine whether mercury-sensitive water channels provided the principal route for water movement in individual alveolar epithelial cells and in the intact lung. Cell and transepithelial lung water transport was rapid and reversibly inhibited by HgCl$_2$, providing strong evidence for transcellular transport of water through mercury-sensitive water channels.

METHODS

Northern Blot and Immunofluorescence. Northern blot analysis was done as described (19) by using a full-length cDNA probe corresponding to the 807-bp coding sequence of rat CHIP28. Blots were hybridized at 42°C for 16 hr with the $^{32}$P-labeled cDNA probe. Immunoblot analysis was done as described (23). Tissue homogenates from exsanguinated rats were resolved on SDS/13% PAGE and blotted to a nitrocellulose membrane. The membrane was blocked, incubated with a purified rabbit anti-CHIP28 antibody, and then incubated with an anti-rabbit IgG-alkaline phosphatase conjugate antibody. For quantitative immunoblotting, six different quantities (0.5-1000 ng) of purified CHIP28 protein (23) were used as standards.

For immunofluorescence, frozen sections of rat and sheep lung were stained with a rabbit polyclonal anti-CHIP28 antibody (11). Sections were blocked in 1% bovine serum albumin/phosphate-buffered saline and incubated with a 1:500 dilution of immune or preimmune serum overnight at 4°C. Slides were then incubated for 1 hr with a secondary fluorescein-conjugated sheep anti-rabbit antibody.

Water Permeability in Alveolar Type II Epithelial Cells. Alveolar type II cells were isolated from pathogen-free Sprague-Dawley rats by digestion with porcine pancreatic

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. $1734 solely to indicate this fact.

Abbreviation: CHIP28, channel-forming integral membrane protein of 28 kDa.

*To whom reprint requests should be addressed.
elastase and selective adhesion plating on rat IgG (28). Cell purity was 85 ± 4% by Papanicolaou staining, cell viability was 92 ± 2% by erythrocyte B dye exclusion, and average cell diameter was 9.1 ± 0.9 μm (SD) by Coulter counting; average cell volume was 382 ± 60 μm³, in agreement with 389 and 366 μm² reported for type II alveolar epithelial cells in situ (29, 30). For immunoblot analysis, a membrane pellet from the type II cells was obtained after homogenization and discarding a low-speed pellet.

Osmotic water permeability (Pᵢ) was measured in freshly suspended alveolar type II cells by a stopped-flow light-scattering technique (15, 31). Cells in Krebs'–Henseleit (KH) buffer (≈8 x 10⁶ cells per ml) were mixed in <1 ms with hyperosmotic solution (KH buffer/800 mM sucrose) to give a 400-mOsm inwardly directed osmotic gradient. The time course of 90° scattered light intensity at 520-nm wavelength was measured. Pᵢ was calculated from the light scattering data and cell surface-to-volume ratio (6590 cm⁻¹, calculated for a 9.1-μm diameter sphere) (31). In some experiments, 0.5 mM HgCl₂ was added at 3 min before measurements.

**Water Permeability in Intact Sheep Lung.** An in situ-perfused sheep lung model was used as modified from our perfused goat lung preparation (32). A total of seven sheep (average weight, 30 kg) were anesthetized, intubated, and ventilated with 100% O₂ (10 mg/kg, tidal volume). The sheep was exsanguinated after infusion of heparin (1000 international units/kg). The perfusate consisted of 1 liter of autologous blood in 500 ml of Ringer lactate.

The chest was opened via a midline sternotomy, and catheters were inserted in the pulmonary artery and left atrium for continuous perfusion. The superior and inferior vena cavae, the ayzygos and hemiazygos veins, and the aorta were ligated. Two distal instillation/withdrawal Silastic catheters were inserted into the lower trachea through needle holes and guided to the distal air spaces of the left and right lungs bronchoscopically. The perfusate was maintained at 20°C to inhibit active Na⁺ transport across the alveolus (31). Sixty milliliters of hyperosmolar instillate containing 2.7% NaCl, 1% sheep albumin, Evans blue dye at 10 mg/liter, 3 μCi (1 Ci = 37 GBq) of 125I-labeled human serum albumin, and 0 or 5 μCi of [14C]mannitol was instilled into the left lung over 2 min. Distal air space samples (1 ml) were obtained every 30–60 s. Then the right lung was instilled with 60 ml of hyperosmotic solution, except that 0.5 mM HgCl₂ was present in the perfusate and instillate, and the instillate contained 131I-labeled albumin instead of 125I-labeled albumin. In some experiments, [14C]mannitol was instilled or 5 mM 2-mercaptopetoethanol was added to the perfusate 5 min after HgCl₂ addition. Sample osmolalities, protein concentrations, and radioactivities were measured. The quantities of the alveolar tracers appearing in the perfusate were calculated from perfusate radioactivity and instillate volume. Data are reported as mean ± SEM. Half-time (τ₁/₂) values were determined by single-exponential regression, and statistical significance was determined by ANOVA.

**RESULTS**

A Northern blot probed by a cDNA corresponding to the coding sequence of rat kidney CHIP28 showed that lung contains an ~2.9-kb mRNA similar to that in kidney (Fig. 1A). mRNA encoding CHIP28 was not detected in brain, liver, muscle, and spleen. Immunoblots probed by an anti-CHIP28 antibody showed large amounts of nonglycosylated (at 28 kDa) and glycosylated CHIP28 protein in rat kidney and lung and human erythrocytes (Fig. 1B). Controls with preimmune serum were negative. CHIP28 protein was also detected in the type II alveolar epithelial cells isolated from rat lung, as shown in Fig. 1C. In three quantitative immunoblots using erythrocyte CHIP28 as standards, there was

![Fig. 1. Expression of CHIP28 water channels in lung. (A) Northern blot probed with 3P-labeled cDNA corresponding to the coding sequence of rat CHIP28. Twenty micrograms of total RNA from the indicated tissues was loaded in each lane. (B) Coomassie blue-stained SDS/PAGE (Left) and immunoblot (Right) showing CHIP28 protein expression in rat kidney and lung. Lanes were loaded with 1 μg of purified erythrocyte CHIP28 (left lanes), 400 μg of rat kidney homogenate (middle lanes), and 200 μg of rat lung homogenate (right lanes). (C) Quantitative immunoblot of membrane proteins isolated from type II alveolar epithelial cells. ~200 ng of CHIP28 per mg of protein in total rat lung homogenate and ~75 ng of CHIP28 per mg of protein in a membrane pellet from the type II alveolar epithelial cells. Cellular localization was studied by immunostaining. Fig. 2A shows that alveolar cells in sheep lung expressed CHIP28 strongly; the section at high magnification (Fig. 2C) suggests staining of alveolar epithelial cells and capillary endothelial cells and some staining of airway epithelial cells. The staining of capillary endothelium is in agreement with a recent study by Nielsen et al. (33). The staining of epithelial cells is consistent with the immunoblot of type II alveolar epithelial cells (Fig. 1C) and functional studies below. The preimmune serum control sections (Fig. 2B and D) showed little staining.

To determine whether the alveolar water channels were functional, stopped-flow light-scattering measurements were done on freshly isolated type II alveolar epithelial cells (Fig. 3A) (the Inset shows the increased curve amplitude with osmotic gradient size). Assuming that the type II cells are perfect osmometers, final relative cell volumes were 0.55, 0.71, 0.83, and 1 in response to osmotic gradients of 250, 125, 62.5, and 0 mM, respectively; corresponding relative signal amplitudes were 1.0, 0.61, 0.36, and 0, indicating a nearly linear relationship between cell volume and scattered light intensity (31). Pᵢ was 0.015 ± 0.002 cm/s (SEM, three preparations) at 10°C.

After incubation with 0.5 mM HgCl₂ (Fig. 3A, second curve) Pᵢ decreased to 0.003 ± 0.001 cm/s. The inhibition by HgCl₂ was fully reversed by 2-mercaptoethanol (Pᵢ, 0.018 ± 0.003 cm/s, third curve). In addition to mercury inhibition, another characteristic of channel-mediated water permeability is a low activation energy (Eₘ) for Pᵢ (34, 35). Fig. 3B shows that the slope of an Arrhenius plot gives an Eₘ of 4 ± 2 kcal/mol. This value is similar to Eₘ in erythrocyte and kidney tubule cell membranes but is much lower than that of >10 kcal/mol in membranes not containing water channels (36).

To determine the role of these cellular water channels in capillary-to-air-space water movement in the intact lung, an in situ perfused sheep lung model was used. Fig. 4 shows the time course of osmotic water movement after instillation of
hypertonic saline into the air spaces. In the control lung, the osmolality decreased from that of the instillate (≈900 mosm) to that of the perfusate (≈300 mosm) with a half-time of 0.85 ± 0.1 min (SE, n = 6, Fig. 4A); the Inset shows a semilogarithmic plot indicating that equilibration of osmolality occurred in an approximately exponential manner. Fig. 4B shows that the dilution of infused radioiodinated albumin paralleled the decrease in air space fluid osmolality. To determine whether capillary-to-air space water transport was mercury-sensitive, 0.5 mM HgCl₂ was added to the perfusate and instillate of the contralateral lung. Fig. 4A and B shows a 69% inhibition of osmotic water permeability; air space osmolality equilibrated with a half-time of 2.7 ± 0.4 min (n = 4). These results suggest that a significant fraction of water movement in lung occurs by a mercury-sensitive transcellular route.

Experiments were done to confirm that HgCl₂ was not toxic in our experiments. First, 2-mercaptoethanol was added after HgCl₂ to test reversibility of HgCl₂ inhibition. The half-time for equilibration of osmolality was 0.7 ± 0.1 min (n = 3) after 2-mercaptoethanol (Fig. 4A), not significantly different from that of the control lung. Analysis of the leakage of small (mannitol) and large (albumin) tracers from air space to perfusate showed no significant difference in permeation between control and HgCl₂-treated lungs. When [¹⁴C]mannitol was added to the instillate, the amount appearing in the perfusate (expressed as percentage instilled) at the end of the experiment was 7.4 ± 0.5% (SD, n = 2) in control lungs and 7.1 ± 4% (n = 3) in HgCl₂-treated lungs. When radiolabeled albumin was added to the instillate, the quantity appearing in the perfusate was 0.6 ± 0.8% (n = 7) in control lungs and 1.5 ± 1.4% (n = 3) in HgCl₂-treated lungs. Further, the movement of native protein from capillary-to-air space was not different for the control (0.1 ± 0.1 g) and HgCl₂-treated (0.2 ± 0.1 g) lungs.

To estimate transalveolar P₀, the air space was taken as a water-permeable alveolar compartment in series with a water-tight "storage" compartment. It was assumed that the instilled fluid was primarily in the alveolar space. Given an alveolar diameter of 200 μm and an instillate volume of 60 ml, ~1.4 × 10⁷ alveoli were fluid-filled, giving an effective surface area for trans-alveolar water transport of ~1.8 × 10⁶ cm². P₀ is defined by Jᵥ = P₀Sᵥw(C₃ - C₄), where Jᵥ is volume

![Fig. 2. Immunostaining of sheep lung with anti-CIP28 antibody (A and C) and preimmune serum control (B and D). Micrographs are shown at low (A and B) and high (C and D) magnifications. Labels denote alveolus (A), alveolar epithelial cell (ae), capillary (cap), and small airway lumen (L). (∼×75 (A and B); ×150 (C and D).)![](image1.png)

![Fig. 3. Osmotic water permeability in freshly suspended type II alveolar epithelial cells. Cells were subjected to inwardly directed gradients of sucrose in a stopped-flow apparatus at 10°C. (A) The time course of scattered-light intensity in response to a 400 mM sucrose gradient. There was significant inhibition of water permeability by 0.5 mM HgCl₂ that was reversed by 5 mM 2-mercaptoethanol (ME). (Inset) Effect of sucrose gradient size on light-scattering curves. (B) Arhenius plot of ln P₀ vs. reciprocal temperature (1/T). Activation energy was equal to 4 ± 2 kcal/mol (1 cal = 4.184 J).](image2.png)
flow into the alveolus (cm\(^3\)/s), \(S\) is surface area (cm\(^2\)), \(v_w\) is partial molar volume of water (18 cm\(^3\)/mol), and \(C_1\) and \(C_2\) are the osmolarities of the air space and perfusate solutions, respectively (35). The parameters were evaluated just after instillation (zero time) to estimate \(P_t\). Substituting \(S = 1.8 \times 10^4\) cm\(^2\), \(C_1 - C_2 = 600\) mOsm, and the measured initial \(J_e\) of 1.9 cm\(^3\)/s, \(P_t\) is 0.010 cm/s at 20°C. Assuming that the apical and basolateral membranes have equal \(P_t\) with \(E_x\) of 4 kcal/mol, the estimated \(P_t\) for individual alveolar epithelial cell plasma membranes is 0.029 cm/s at 37°C.

**DISCUSSION**

Northern blots and quantitative immunoblots indicated high expression of CHIP28 mRNA and protein in lung. Immunocytochemistry localized CHIP28 primarily to alveolar epithelial–cell plasma membranes, with some staining of alveolar capillary endothelial cells and airway epithelial cells. Functional studies were done to define the role of mercury-sensitive water channels in the transport of water between the airway and capillary compartments. \(P_t\) in alveolar type II epithelial cells was high, weakly temperature-dependent, and reversibly inhibited by HgCl\(_2\). Osmotically driven water transport from capillary-to-air space in the in situ sheep lung model was rapid and inhibited reversibly by HgCl\(_2\). Because HgCl\(_2\) cannot inhibit paracellular water transport, the inhibition of capillary-to-air-space water permeability by HgCl\(_2\) indicates that water movement in lung was transcellular and mediated by mercury-sensitive water channels.

A concern in water-transport measurements in the in situ-perfused lung was the presence of unstirred layers that might limit the rate of water transport (37). Unstirred layers could arise from poorly mixed aqueous compartments in the air space, alveolar epithelial cell, and/or capillary interstitium. Although intracellular unstirred layers cannot be eliminated, interstitial unstirred layers were minimized by continuous vascular perfusion, and air space unstirred layers were minimized by inducing high osmotic water flow from capillary-to-air space to produce continuous volume flow into the air spaces. The inhibition of water movement in the intact lung by HgCl\(_2\) and the high transepithelial \(P_t\) of 0.01 cm/s provided evidence that little or no unstirred layer effects were present under the conditions of the experiment.

The high \(P_t\) values measured in the isolated alveolar epithelial type II cells and in the intact lung suggest that alveolar-cell plasma membranes contain abundant quantities of functional water channels. Using a plasma membrane \(P_t\) value of 0.03 cm/s (extrapolated to 37°C) and a single-channel water permeability for CHIP28 of \(10^{-12}\) cm\(^2\)/s measured in reconstituted proteoliposomes (15), the plasma-membrane density of water channels is \(3 \times 10^{11}\) water channels per cm\(^2\). Assuming that the plasma membranes are 50% protein by weight, then it is estimated that water channels comprise \(\approx 5%\) of total plasma membrane protein. This high value is consistent with the high abundance of CHIP28 protein by quantitative immunoblot. Assuming that the majority of CHIP28 protein is located at the plasma membrane of alveolar epithelial cells (each with average surface area \(1.4 \times 10^{-6}\) cm\(^2\), ref. 30), then each cell should contain \(\approx 2 \times 10^{-11}\) mg of CHIP28. Assuming that the alveolar epithelial cells make up \(\approx 50%\) of total lung weight (29) and an average alveolar epithelial-cell protein content of \(2 \times 10^{-7}\) mg, it is estimated that the lung should contain \(\approx 100\) mg of CHIP28 per mg of protein. Recognizing uncertainties in the amount of intracellular CHIP28 (11, 26) and other water channels (13), this value is similar to that of 200 ng per mg of protein determined by quantitative immunoblot.

The inhibition of capillary-to-air space water movement by HgCl\(_2\) in intact lung indicated transcellular water transport. In other epithelia, evidence for transcellular water transport has included (i) inhibition of proximal tubule water transport by mercurials (38), (ii) comparative permeabilities of water and a series of small polar solutes (39), and (iii) theoretical calculations of water permeability from the morphology of the paracellular space (40). The transcellular route for lung water movement found here is consistent with the high expression of water channels in alveolar epithelial cell plasma membranes. Interestingly, almost 20 yr ago, Effros (41) suggested that the hypertonic extraction of water from lung tissue occurs by a transcellular route, although the existence of specific water channels was not recognized at that time.

Our studies demonstrated that the route for capillary-to-air space water transport in response to relatively large osmotic gradients was transcellular and involved mercury-sensitive water channels. Does this conclusion apply in vivo where osmotic gradients are small and there may be hydrostatic as well as osmotic forces, as in the resolution of alveolar edema from the air spaces of the lung? A fundamental result of nonequilibrium thermodynamics (42) is that if the properties of the barrier to water movement are constant, then the water permeability determined for a single osmotic gradient would apply to all osmotic and hydrostatic gradients. One situation in which the barrier to water movement might change is paracellular water transport across an epithelium, in which intercellular geometry depends on osmotic gradient size (43); however, this situation would not apply in lung, where the
route for water movement is transcellular. Therefore, we believe that trans-alveolar water movement is primarily transcellular under normal physiological conditions.

We propose that an important role for lung water channels is to facilitate the near-isotonic transport of water across the alveolar epithelium. A water-permeable epithelial layer is required to produce net fluid transport in response to the small osmotic gradients established by salt transport. Another important reason for water movement from capillary-to-air space is the replacement of solute-free water to offset evaporative losses that accompany respiration. Although humidification of inspired air occurs in airways rather than in alveoli, water may move across the bronchial epithelial layer or across the alveolar epithelial layer and then into the airways. Physiological data are not available to distinguish between these possibilities.

The immunocytochemical results indicate high expression of CHIP28 water channels in alveolar epithelial cells. Although type II alveolar epithelial cells have been shown to transport Na⁺ actively (6, 8), the only established function of the more prevalent alveolar epithelial type I cell involves the formation of tight junctions to maintain epithelial integrity (44). The high expression of CHIP28 water channels in the type I cells found here suggests that a major role of the type I cells is to transport water. In the thin descending limb of Henle in mammalian kidney, the epithelial cells are also highly water-permeable but have relatively low metabolic activity and lack other distinct functions (45). We propose that a primary function of alveolar type I cells, as well as epithelial cells in the thin descending limb, is to provide a selective water-permeable cell layer.

We thank Drs. Alfred van Hoek and Antonio Frigeri for help in immunoblot analysis. This work was supported by Grants HL25816, DK35124, and HL42368 from the National Institutes of Health and a grant from the National Cystic Fibrosis Foundation. Dr. Hasegawa is a fellow of the National Kidney Foundation; Dr. Folkesson was supported by Grant HL19155.