

# Airway Surface Liquid Osmolality Measured using Fluorophore-encapsulated Liposomes

SUJATHA JAYARAMAN,\*<sup>†</sup> YUANLIN SONG,\*<sup>†</sup> and A.S. VERKMAN\*<sup>†</sup>

From the \*Department of Medicine and <sup>†</sup>Department of Physiology, Cardiovascular Research Institute, University of California, San Francisco, San Francisco, California, 94143

**ABSTRACT** The airway surface liquid (ASL) is the thin layer of fluid coating the luminal surface of airway epithelial cells at an air interface. Its composition and osmolality are thought to be important in normal airway physiology and in airway diseases such as asthma and cystic fibrosis. The determinants of ASL osmolality include epithelial cell solute and water transport properties, evaporative water loss, and the composition of secreted fluids. We developed a noninvasive approach to measure ASL osmolality using osmotically sensitive 400-nm-diam liposomes composed of phosphatidylcholine/cholesterol/polyethylene glycol-phosphatidylcholine (1:0.3:0.08 molar ratio). Calcein was encapsulated in the liposomes at self-quenching concentrations (30 mM) as a volume-sensitive marker, together with sulforhodamine 101 (2 mM) as a volume-insensitive reference. Liposome calcein/sulforhodamine 101 fluorescence ratios responded rapidly (<0.2 s) and stably to changes in solution osmolality. ASL osmolality was determined from calcein/sulforhodamine 101 fluorescence ratios after addition of microliter quantities of liposome suspensions to the ASL. In bovine airway epithelial cells cultured on porous supports at an air-liquid interface, ASL thickness (by confocal microscopy) was 22  $\mu\text{m}$  and osmolality was  $325 \pm 12$  mOsm. In anesthetized mice in which a transparent window was created in the trachea, ASL thickness was 55  $\mu\text{m}$  and osmolality was  $330 \pm 36$  mOsm. ASL osmolality was not affected by pharmacological inhibition of CFTR in airway cell cultures or by genetic deletion of CFTR in knockout mice. ASL osmolality could be increased substantially to >400 mOsm by exposure of the epithelium to dry air; the data were modeled mathematically using measured rates of osmosis and evaporative water loss. These results establish a ratio imaging method to map osmolality in biological compartments. ASL fluid is approximately isosmolar under normal physiological conditions, but can become hyperosmolar when exposed to dry air, which may induce cough and airway reactivity in some patients.

**KEY WORDS:** water permeability • cystic fibrosis • fluorescence self-quenching • trachea • CFTR

## INTRODUCTION

The air-bathed surfaces of airways in the nasopharynx, trachea, and distal bronchi represent a unique biological air-fluid interface. The luminal (air-facing) surface of airway epithelia contains a thin (20–75  $\mu\text{m}$ ) layer of fluid called the airway surface liquid (ASL).<sup>1</sup> The amount of water, salts, osmolytes, and macromolecules in the ASL depends on the transporting properties of the airway epithelia, evaporative water losses, macromolecular secretion, and possibly other factors. The airways in vivo also contain beating cilia, producing convective and surface tension phenomenon, as well as submucosal glands that can secrete fluid and macromolecules onto the airway surface. The ASL could in principle be hyposmolar, isosmolar, or hyperosmolar (compared with blood osmolality) depending on the relative influence of these factors. For example, a hy-

per tonic ASL might result from evaporative water losses across a relatively water-impermeable barrier, whereas a hypotonic ASL might result from avid ion absorption or possibly surface tension phenomena (Boucher, 1994, 1999; Quinton, 1994; Widdicombe et al., 1997).

There is conflicting evidence that the ASL is hypotonic under normal conditions and becomes near isotonic in the inherited disease cystic fibrosis (CF; Gilljam et al., 1989; Joris et al., 1993; Smith et al., 1996; Zabner et al., 1998). An increase in ASL salinity in CF has been proposed to inhibit endogenous antimicrobials and promote lung infection (Goldman et al., 1997; Zabner et al., 1998). Changes in ASL osmolality are proposed to play a role in the pathophysiology of asthma and cough, wherein local irritation stimulates neurogenic reflexes (Hahn et al., 1984; Higenbottam, 1984; Daviskas et al., 1996). The composition of the ASL has been difficult to measure because of its small volume. Invasive sampling methods involving filter paper or microcapillary fluid collections have yielded a wide range of ASL salt concentrations (Gilljam et al., 1989; Erjefalt and Persson, 1990; Joris et al., 1993; Smith et al., 1996; Goldman et al., 1997; Knowles et al., 1997; Hull et al., 1998; Zabner et al., 1998; Baconnais et al., 1999; Cowley et al., 2000). In-

Address correspondence to Alan S. Verkman, M.D., Ph.D., 1246 Health Sciences East Tower, Cardiovascular Research Institute, University of California, San Francisco, San Francisco, CA 94143-0521. Fax: (415) 665-3847; E-mail: verkman@itsa.ucsf.edu

<sup>1</sup>Abbreviations used in this paper: ASL, airway surface liquid; CF, cystic fibrosis; PC, phosphatidylcholine; PEG, polyethylene glycol.

vative sampling methods have been criticized because of local irritation produced by mechanical stimulation, as well as the inappropriate sampling of cellular and interstitial fluids by capillary action (Erjefalt and Persson, 1990; Widdicombe et al., 1997; Boucher, 1999; Pilewski and Frizzell, 1999; Wine, 1999).

We recently introduced noninvasive fluorescence methods to measure ASL  $[Na^+]$ ,  $[Cl^-]$ , and pH in airway cell culture models and in the in vivo trachea (Jayaraman et al., 2001). The ASL is stained with polar ion- or pH-sensitive fluorescent indicators for ratio imaging microscopy. We found that ASL  $[Na^+]$  and  $[Cl^-]$  were in the range 90–120 mM in airway cell culture models and 100–140 mM in mouse trachea. However, it was not possible to determine ASL osmolality because  $[K^+]$  and other potentially important cations and anions were not measured. Thus, it remains unknown whether the ASL is hyposmolar, isosmolar, or hyperosmolar, whether ASL osmolality differs in cystic fibrosis, and whether ASL osmolality can be increased by ventilation with dry air.

The purpose of this study was to measure ASL osmolality noninvasively. Because fluorescent osmolality-sensitive indicators do not exist, we designed a ratioable fluorescent osmotic sensor based on osmotically driven changes in liposome volume. Liposome composition and size were optimized to minimize interactions with the cell surface and other components of the ASL, and to permit rapid osmotic equilibration without solute equilibration. The inclusion of polyethylene glycol (PEG)-phosphatidylcholine (PC) and cholesterol in the lipid mixture was required to satisfy these requirements. Polar fluorophores (calcein and sulforhodamine 101) were selected that could be encapsulated stably in the liposomes and give a bright, ratioable, osmolality-sensitive fluorescent signal. Local ASL osmolality could be measured to better than 10 mOsm accuracy in the physiological range with this approach. We report the first measurements of ASL osmolality and provide direct evidence that the ASL is normally near isosmolar and not different in cystic fibrosis. In addition, we have evaluated experimentally and theoretically the influence of evaporative water loss and dry air breathing on ASL osmolality.

## MATERIALS AND METHODS

### Preparation of Liposomes

PC (20 mg), cholesterol, and PEG-PC in the molar ratio 1:0.3:0.08 from chloroform stock solutions were mixed, and the chloroform was evaporated to give a thin lipid film. Multilamellar vesicles were prepared by hydrating the lipid film with 1 ml of buffer containing 1 mM  $CaCl_2$ , 0.5 mM  $MgCl_2$ , 2.7 mM KCl, 1.47 mM  $KH_2PO_4$ , 15 mM  $Na_2HPO_4$ , 105 mM NaCl, 2 mM Tris, 30 mM calcein, and 2 mM sulforhodamine 101, pH 7.4. Solution osmolalities were adjusted to 200 or 300 mOsm (with NaCl) as required, and measured on a  $\mu$ -Osmette osmometer (Precision Instruments Inc.). For preparation of small uniformed size liposomes,

the vesicle suspension was freeze-thawed four times and passed through 400-nm pore size polycarbonate membrane filters (Avanti Polar Lipids Inc.) using an Avanti mini-extruder. Unencapsulated dye was removed by size exclusion chromatography using a column (Sephadex G-50; Sigma-Aldrich). Suspensions of liposomes were kept at 4°C until use.

### Steady-state and Stopped-flow Fluorescence Measurements

Steady-state fluorescence titrations were carried out on a fluorescence plate reader (Fluostar; BMG Labtechnologies Inc.) equipped with temperature regulation and excitation/emission filter sets for calcein (480/530 nm) and sulforhodamine 101 (590/620 nm). Microliter aliquots of stock liposome suspensions were added to solutions containing specified concentrations of NaCl, sucrose, glucose, lactose, or urea. Stopped-flow fluorescence measurements were performed with a Hi-Tech stopped flow apparatus with instrument deadtime <2 ms and 25 kHz data acquisition rate. 0.01 ml of the liposome suspension (0.5 mg lipid/ml) was mixed with an equal volume of buffer to give specified solute gradients. Fluorescence was excited at 480 nm and collected using a >520 cut-on filter. All measurements were done at 37°C.

### Cell Culture Experiments

Bovine tracheal cells were cultured on collagen-coated 12-mm-diam snapwell inserts (Costar) with polycarbonate semipermeable membranes at an air-liquid interface at 37°C in a 5%  $CO_2$  / 95% air atmosphere until fully differentiated as described by Uyekubo et al. (1998). Culture medium was changed every 2–4 d. Cultures

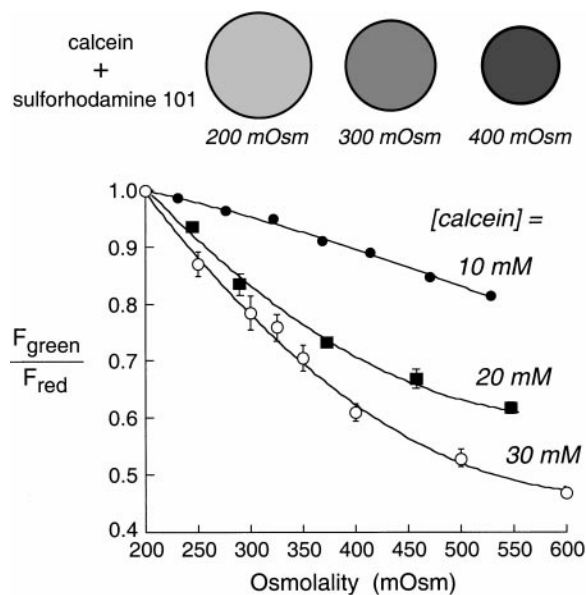


FIGURE 1. Strategy to measure ASL osmolality using volume-sensitive fluorescent liposomes. (top) Liposomes encapsulating calcein and sulforhodamine 101 were prepared by extrusion and exclusion chromatography. When exposed to increasing external solution osmolalities, liposome volume decreases osmotically, green calcein fluorescence decreases (because of self-quenching), and red sulforhodamine 101 fluorescence does not change. (bottom) Ratio of calcein green fluorescence to sulforhodamine 101 red fluorescence ( $F_{green}/F_{red}$ ; mean  $\pm$  SEM,  $n = 3$ ) as a function of solution osmolality. Liposomes were prepared in 200 mOsm buffer in the presence of indicated [calcein] and increasing solution osmolalities, as established by the addition of sucrose.

were generally used 25–30 d after plating, at which time the electrical resistance was  $>300 \Omega\text{cm}^2$  and transepithelial potential difference was  $>20$  mV. Cell inserts were mounted (cells facing upward) in a stainless steel perfusion chamber in which the undersurface of the insert was perfused with solutions at specified rates and perfusion pressures. The chamber was maintained at  $37^\circ\text{C}$  using a PDMI-2 microincubator (Harvard Apparatus), positioned on the stage of an upright epifluorescence microscope, and enclosed in a 100% humidified air/5%  $\text{CO}_2$  tent maintained at  $37^\circ\text{C}$ . For depth measurements, the ASL was stained with tetramethylrhodamine-dextran dispersed in a low boiling point perfluorocarbon (boiling point  $56^\circ\text{C}$ ; Fluorinert FC-72; 3M Co.) as described previously (Jayaraman et al., 2001). For osmolality measurements, 46 nl of the liposome suspension (5 mg lipid/ml suspended in PBS containing 2 mM  $\text{CoCl}_2$ ) was deposited onto the ASL using a microinjector (Nanoject-II; Drummond Scientific Co.).

### Fluorescence Microscopy

Fluorescence was measured using a Nipkow wheel-type confocal microscope (Leitz with Technical Instruments confocal/coaxial module) with photomultiplier detector, cooled CCD camera (Photometrics), and custom filter sets (Chroma) for tetramethylrhodamine, calcein, and sulforhodamine 101. Fluorescence was detected using a Nikon  $50\times$  extralong working distance air objective (numerical aperture 0.55, working distance 8.5 mm). For osmolality measurements, calcein and sulforhodamine 101 fluorescence were recorded at specified times after application of the liposome suspension. Background fluorescence was under 1% of measured fluorescence in all measurements. For ASL thickness measurements, the microscope fine focus was driven by a microstepper motor to record the axial fluorescence profile as the focal plane moved through the ASL volume. ASL thickness was determined to better than  $2 \mu\text{m}$  accuracy using a deconvolution technique as described previously (Jayaraman et al., 2001).

### Measurements in Mouse Trachea In Vivo

Mice (25–35 g body weight, wild-type and Cambridge CF null mice; CFRDP Transgenic Core Facility) were anesthetized with pentobarbital (50 mg/kg, intraperitoneal) 10 min after pretreatment with atropine (1 mg/kg intraperitoneal). As described previously (Jayaraman et al., 2001), atropine was used to avoid induction of gland secretions by the surgical procedure that was seen in 10–25% of untreated mice. A midline incision was made in the neck to expose the trachea for measurement of fluorescence through the translucent tracheal wall. The ASL was stained by instillation of 0.5–1  $\mu\text{l}$  of the liposome suspension using a microcatheter inserted into a feeding needle introduced via the mouth. The mouse was positioned on the microscope stage for fluorescence measurements as described above. In some experiments, mice breathed dry air through a cannula inserted via a tracheostomy. After instillation of the liposome suspension through the cannula, fluorescence was measured 2–3 and 9–12 mm distal to the tip of the cannula. After completion of the measurements, mice were killed by an overdose of pentobarbital (150 mg/kg). Animal protocols were approved by the UCSF Committee on Animal Research.

## RESULTS

Fig. 1 (top) depicts the strategy for measurement of osmolality by ratio imaging microscopy. Liposomes of 400-nm diameter composed of PC, PEG-PC, and cholesterol were generated by extrusion in the presence of calcein and sulforhodamine 101. After chromatographic separation from external fluorophore, the dye-

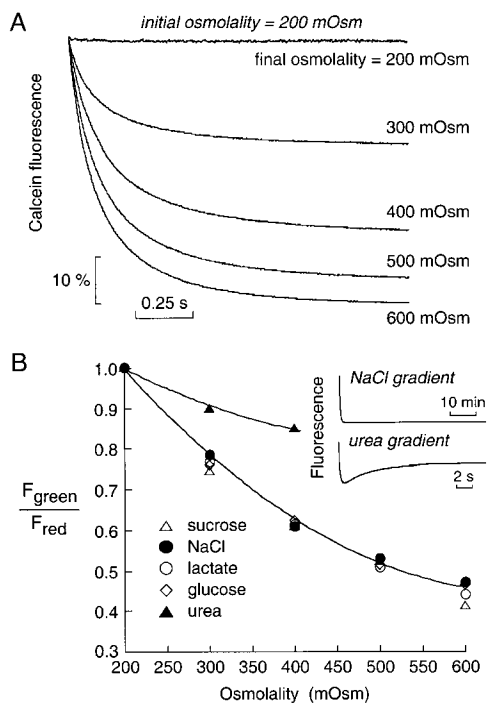
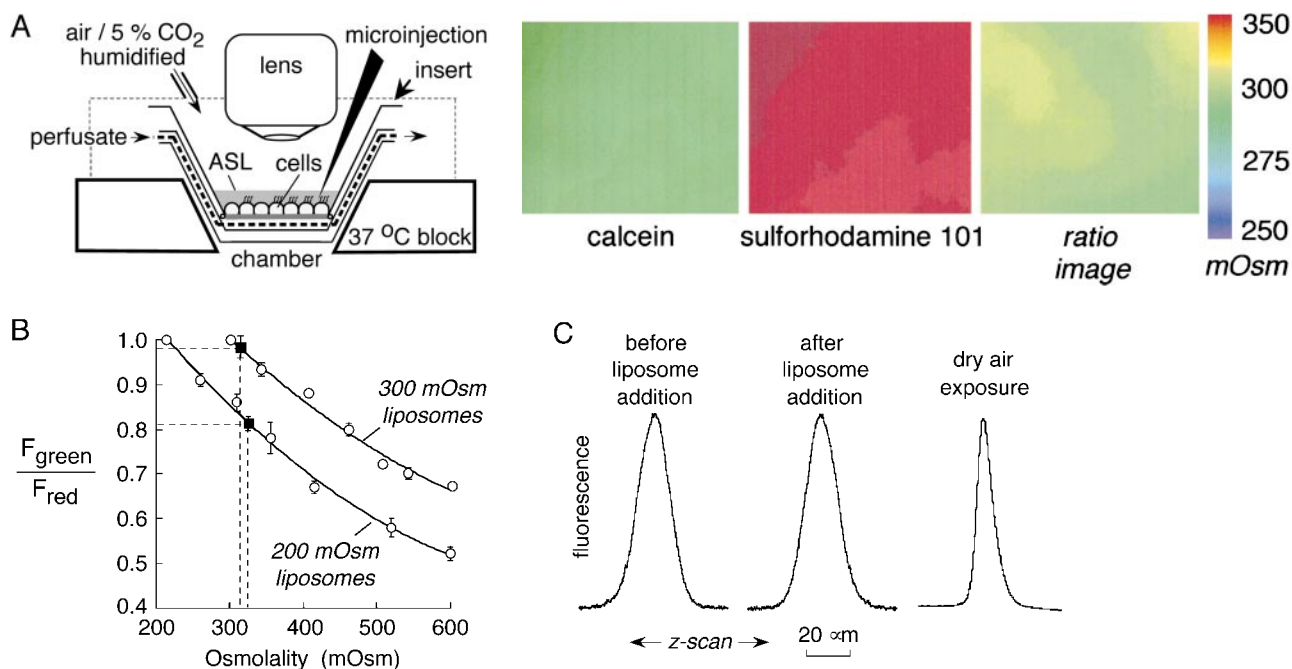


FIGURE 2. Characterization of osmotically sensitive liposomes. (A) Stopped-flow fluorescence quenching of calcein fluorescence. 200-mOsm liposome suspensions were mixed with an equal volume of phosphate buffer containing excess NaCl to give the indicated final osmolalities. Calcein fluorescence decreases because of self-quenching as liposome volume decreases. (B)  $F_{\text{green}}/F_{\text{red}}$  for liposomes suspended in PBS in which osmolality was increased to 400 mOsm by addition of sucrose, NaCl, lactate, glucose, or urea as indicated. Suspensions were incubated for 1 h before measurements. (inset) Time course of calcein fluorescence in liposomes mixed with PBS containing 400 mOsm NaCl (top) or urea (bottom) measured by stopped-flow.

encapsulated liposomes functioned as a ratioable osmotic sensor. Increasing external osmolality resulted in osmotic shrinkage and increased dye concentration. The green fluorescence of calcein decreases because of self-quenching, whereas the red fluorescence of sulforhodamine 101 does not change. As shown in Fig. 1 (bottom), the green-to-red fluorescence ratio ( $F_{\text{green}}/F_{\text{red}}$ ) decreased with increasing external osmolality. Liposome size, lipid composition, fluorescent dyes, and dye concentrations were optimized to give stable osmolality-sensing liposomes that interacted minimally with cell membranes and ASL constituents (see DISCUSSION). The dependences of  $F_{\text{green}}/F_{\text{red}}$  on external osmolality for different [calcein] are shown; 30 mM calcein was used for subsequent measurements.

The osmolality-sensing liposomes were further characterized. Size analysis by quasi-elastic light scattering indicated a unimodal size distribution with liposome diameter  $382 \pm 4$  nm. Liposome osmotic water permeability was measured by stopped-flow fluorescence quenching. Fig. 2 A shows the time course of calcein



**FIGURE 3.** ASL osmolality in monolayer cultures of airway epithelial cells. (A, left) Schematic of apparatus for measurement of liposome fluorescence in ASL. Snapwell inserts containing cell monolayers grown at an air–liquid interface were mounted in a perfusion chamber enclosed in a 100% humidity, 5% CO<sub>2</sub> tent. (A, right) Images of liposome-stained ASL recorded using calcein (green) and sulforhodamine 101 (red) filter sets, shown with pseudocolored ratio image with osmolality scale. (B)  $F_{\text{green}}/F_{\text{red}}$  measured by fluorescence microscopy for liposomes prepared in 200- or 300-mOsm solutions and suspended in solutions of different osmolalities (open circles, SEM,  $n = 3$ ). The same liposomes were added to the ASL (closed squares) of cell cultures ( $n = 7$ – $9$  cultures). (C) Determination of ASL depth by z-scanning confocal microscopy as described in MATERIALS AND METHODS. Representative z-scans for tetramethylrhodamine-stained ASL before (left) and after (middle) addition of the liposome suspension, and after exposure to dry air for 10 min (right). See text for averaged ASL depths.

fluorescence in response to a sudden change in solution osmolality to different values (200–600 mOsm) as indicated. Osmotic equilibration was >95% complete in under 1 s, with a computed osmotic water permeability coefficient ( $P_f$ ) of 0.006 cm/s. To determine whether liposome volume remained stable after osmotically induced volume changes, the time course of calcein fluorescence was measured in response to increasing solution osmolality with different solutes, including the impermeable solute sucrose, and the biologically important solutes NaCl, lactate, glucose, and urea. Fig. 2 B shows stable liposome volume for at least 1 h in response to gradients of NaCl, lactate, and glucose, as shown by the identical  $F_{\text{green}}/F_{\text{red}}$  versus osmolality relationships. As expected, urea equilibrated rapidly within a few seconds, as measured directly by stopped-flow light scattering (Fig. 2 B, inset). Fortunately, except in the kidney or in renal failure, biological urea concentrations are generally <7 mM (10 mg/dl). Dye leakage from liposomes was measured from the time course of calcein fluorescence in the absence of an osmotic challenge. There was <1% increase in fluorescence over 1 h (not shown), with no significant decrease in fluorescence after the addition of 5 mM cobalt (as CoCl<sub>2</sub>), a membrane-impermeable static quencher of calcein fluorescence (Wallach and Steck, 1963). Together with

measurements done over longer times, it was concluded that both dyes leaked out of liposomes by <5% in 5 h.

Measurements of ASL osmolality were done in cultures of bovine airway epithelial cells grown at an air–liquid interface. Fig. 3 A shows the experimental setup along with images of the ASL recorded using the calcein and sulforhodamine 101 filter sets. The pseudocolored ratio image (Fig. 3 A, right) shows a quite uniform osmolality over the ASL surface. Fig. 3 B shows calcein/SR fluorescence ratios measured for liposomes prepared in 200 or 300 mOsm buffers. Calibration values were obtained by suspending liposomes in solutions of indicated osmolalities and measuring fluorescence ratios in an identical manner as done for cells. ASL osmolalities were  $325 \pm 12$  mOsm (SEM;  $n = 8$  cultures) using the 200-mOsm liposomes, in agreement with the value of  $310 \pm 14$  mOsm ( $n = 5$ ) measured using the 300-mOsm liposomes. To show that addition of liposomes did not change ASL volume, the ASL thickness was measured by confocal microscopy just before versus after liposome addition. Fig. 3 C shows representative fluorescence z-scans with average ASL thicknesses of  $22 \pm 3$  μm ( $n = 3$ ) before (left) and  $24 \pm 5$  μm after (middle) addition of the liposome suspension. For comparison, an ASL z-scan is shown at 15 min after ex-

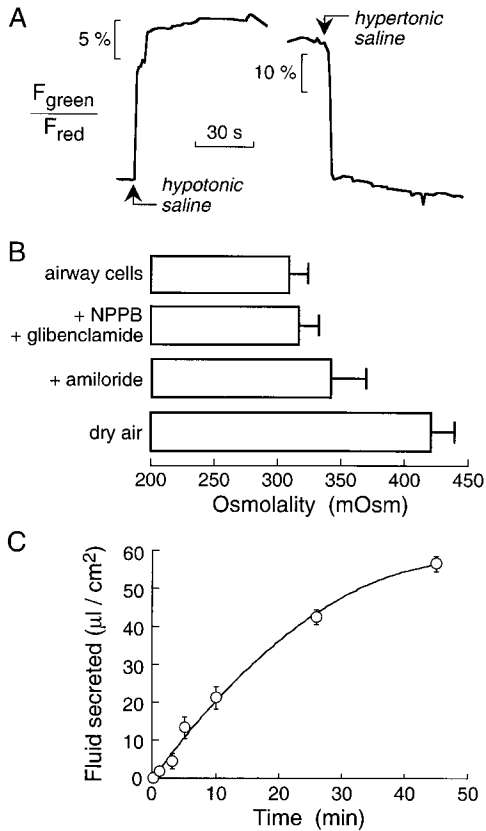


FIGURE 4. ASL osmolality and water flow in response to osmotic challenge in bovine airway cells cultured on porous supports at an air-liquid interface. (A) Time course of  $F_{\text{green}}/F_{\text{red}}$  after addition to the ASL of 20  $\mu\text{l}$  of hypotonic PBS (150 mOsm, left) or hypertonic PBS (500 mOsm, right). (B) ASL osmolalities (SE,  $n = 3-5$  cultures) measured 1 h after incubation with 10  $\mu\text{M}$  amiloride or 100  $\mu\text{M}$  NPPB + 500  $\mu\text{M}$  glibenclamide added to both the serosal and luminal solutions, or after a 5-min exposure to dry (0% moisture content) air. (C) Transepithelial osmotic water permeability in airway cell monolayers. Monolayers on porous supports were subjected to an osmotic gradient by the addition of 200  $\mu\text{l}$  of 600 mOsm buffer containing NaCl and a volume marker (blue dextran). Osmotically induced fluid secretion (microliter per square centimeter of culture) was measured by dye dilution. See text for computed water permeability coefficients.

posure to dry air in which ASL thickness decreased to 8  $\mu\text{m}$  because of evaporative water loss.

Various maneuvers were carried out to change ASL osmolality. Fig. 4 A shows control experiments in which small volumes of hypotonic or hypertonic saline were added to the ASL. As expected, calcein fluorescence and apparent ASL osmolality changed promptly. Fig. 4 B summarizes ASL osmolalities measured under control conditions, in the presence of the transport inhibitors glibenclamide and NPPB (to inhibit CFTR) and amiloride (to inhibit ENaC) and after exposure to dry air. ASL osmolality was not significantly changed by the transport inhibitors, but was increased considerably by rapid evaporative water loss resulting from exposure to dry air.

Because of the important role of osmosis in maintain-

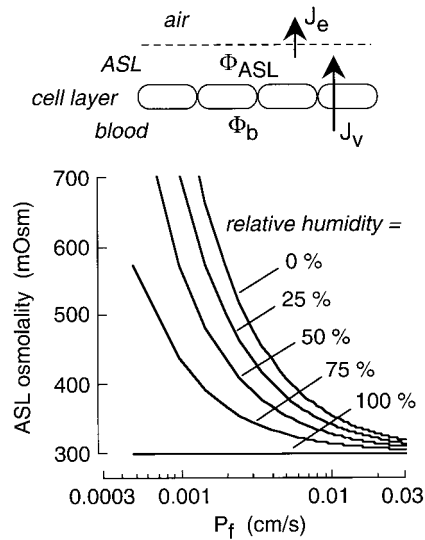
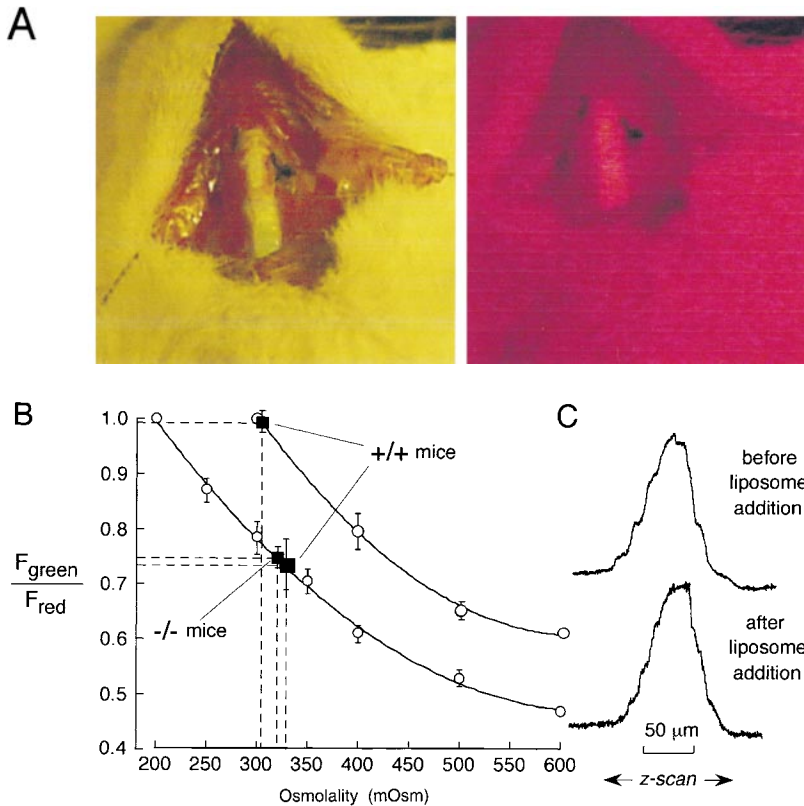


FIGURE 5. Theoretical dependence of ASL osmolality on transepithelial water permeability ( $P_f$ ) and moisture content (relative humidity) of air in contact with the ASL. (top) Schematic of geometry.  $J_e$ , evaporative water loss;  $J_v$ , osmotic volume flow;  $\Phi_{\text{ASL}}$  and  $\Phi_b$ , osmolalities of ASL and basal fluid, respectively. (bottom) Curves computed as described in the text assuming impermeant solutes in the ASL and neglecting convective effects. See RESULTS for explanations.

ing ASL osmolality when evaporative water loss occurs, we measured osmotic water permeability across airway cell layers from the time course of ASL volume after an osmotic challenge. 200  $\mu\text{l}$  of hypotonic saline (150 mOsm) or hypertonic saline (600 mOsm) containing the volume marker blue dextran (0.1 g/ml) was added to apical surface of the cell monolayer. 10- $\mu\text{l}$  aliquots of luminal fluid were removed at specified times for determination of volume marker concentration. Fig. 4 C shows the time course of fluid secretion after adding hypertonic saline to ASL. The computed transepithelial osmotic water permeability coefficient ( $P_f$ ), assuming a smooth epithelial cell surface, was  $(5.5 \pm 1.2) \times 10^{-3}$  cm/s ( $n = 4$  cultures) for induced fluid secretion and  $(6.2 \pm 2.0) \times 10^{-3}$  cm/s for induced fluid absorption (hypotonic saline added to ASL). These values are lower than that of 0.022 cm/s reported in cultures of human airway cells (Matsui et al., 2000).

The osmotic water permeability of the cell layer sets limits on the theoretical magnitude of the difference between ASL and perfusate osmolalities. As depicted in Fig. 5 (top), water is lost from the ASL by evaporation ( $J_e$ ) and replaced from the tracheal wall by osmosis ( $J_v$ ). Neglecting surface tension and convective effects, and assuming no transport of osmotic solutes out of the ASL,  $J_e$  and  $J_v$  are equal in the steady state. ASL osmolality ( $\Phi_{\text{ASL}}$ ) is then calculated from the equation:  $J_e = J_v = P_f v_w S (\Phi_{\text{ASL}} - \Phi_b)$ , where  $v_w$  is the partial molar volume of water (18  $\text{cm}^3/\text{mol}$ ),  $S$  is surface area, and  $\Phi_b$  is perfu-



**FIGURE 6.** ASL osmolality in mouse trachea. The ASL was stained with fluorescent liposomes by instillation of 0.5–1  $\mu$ l of the liposome suspension through a catheter inserted into a feeding needle passed via the mouth. (A) Photographs of exposed trachea showing green calcein (left) and red sulforhodamine 101 (right) fluorescence seen through the translucent tracheal wall. (B)  $F_{green}/F_{red}$  of liposomes (initial osmolalities indicated) measured by fluorescence microscopy in solution of known osmolalities (open circles) and in the ASL (closed squares) (SEM,  $n = 5$ ). (C) ASL depth measured by z-scanning confocal microscopy before and after instillation of the liposome suspension.

sate/serum osmolality. Evaporative water loss ( $J_e$ ) during exposure to dry air was measured gravimetrically to be  $1.1 \times 10^{-5}$  microliters water loss per centimeter squared area, and independent of solution salt content (0–500 mM NaCl). Fig. 5 (bottom) shows the predicted dependence of ASL osmolality on  $P_f$  and relative humidity, showing the expected increase in ASL osmolality with decreasing  $P_f$ . Using the measured  $P_f$  of  $5.5 \times 10^{-3}$  cm/s and dry air (0% humidity), the predicted ASL osmolality is 403 mOsm, in good agreement with the measured value of  $419 \pm 17$  mOsm from Fig. 4 B. This simple model provides a quantitative description of the quasi-steady-state increase in ASL osmolality after exposure to dry air, but is probably inaccurate for long exposure times because of ASL solute transport driven by the increased solute concentration.

Cultured airway cell monolayers represent an imperfect model of the intact airway epithelium because they are not exposed to time-varying air flows with changing moisture, oxygen, and carbon dioxide content. In addition, the intact airways have a considerably more complex structure than uniform monolayers of cultured cells. Therefore, we measured ASL osmolality in the mouse trachea in vivo. Microliter aliquots of liposome suspension were instilled into the lower trachea using a small catheter introduced through a feeding needle. The liposomes rapidly dispersed throughout the trachea. Fig. 6 A shows the fluorescently stained exposed trachea as seen using calcein (green) and SR (red) fil-

ter sets. Fig. 6 B shows  $F_{green}/F_{red}$  values for liposomes in solutions of known osmolalities (open circles) and in the trachea (closed squares). ASL osmolality was  $330 \pm 36$  mOsm ( $n = 5$ ) in wild-type mice, not significantly different from  $321 \pm 27$  mOsm in cystic fibrosis (CFTR-null) mice. ASL osmolalities measured using liposomes prepared in 200 and 300 mOsm buffers were not significantly different. Additional studies were done on wild-type mice in which a rectangular window was created in the anterior tracheal wall to visualize the posterior tracheal mucosa through a transparent plastic window as described previously (Jayaraman et al., 2001). ASL osmolalities measured through the window did not differ significantly from those measured without the window, and ASL depth (range 50–68  $\mu$ m) was not affected by instillation of the liposome suspension (Fig. 6 C). Further studies using intravenously administered FITC-dextran (10 kD) showed that the tracheal wall did not become leaky to macromolecules under the conditions of our experiments.

Further studies were done on wild-type mice to determine whether the ASL could become hyperosmolar when exposed to dry air. Mice were allowed to breath dry air (0% humidity) through a tracheal cannula for 10 min before measurement of ASL osmolality. This maneuver mimics tracheostomy breathing, and is probably a good model for the ASL in the nasopharynx under normal ventilation conditions. We found a significant increase in ASL osmolality to  $438 \pm 11$  mOsm at

2–3 mm distal to the tip of the cannula, decreasing to  $340 \pm 10$  mOsm at 9–12 mm distal to the tip of the cannula in mice ventilated with dry air.

#### DISCUSSION

The fluorescent liposome sensor developed here provided a ratioable signal to map osmolality in macroscopic or microscopic biological compartments. The liposome sensor was applied to important issues in airway physiology—the absolute osmolality of the ASL, and the hypotheses that ASL osmolality is altered in cystic fibrosis and can be increased by exposure to dry air. The methodologies for fluorescent staining of the ASL in cell culture models and intact mouse trachea were recently developed and validated in measurements of ASL  $[\text{Na}^+]$ ,  $[\text{Cl}^-]$ , and pH (Jayaraman et al., 2001). The principal conclusions of this study are that, under normal conditions, ASL osmolality is approximately isosmolar with external osmolality; that pharmacological inhibition or genetic knockout of CFTR in cystic fibrosis does not result in altered osmolality; and that exposure to dry air can substantially increase ASL osmolality. These results extend previous findings that ASL  $[\text{Na}^+]$  and  $[\text{Cl}^-]$  are each  $>100$  mM, that  $[\text{HCO}_3^-]$  is  $\sim 8$  mM, and that these concentrations do not differ significantly in CF. The remaining unmeasured cation to account for the osmolar gap is probably  $\text{K}^+$ , and the remaining unmeasured anions probably include proteins and small organic anions. Our results indicate that substantial quantities of unmeasured osmolytes are not present in the ASL, and that surface tension phenomena do not contribute significantly to osmotic balance.

The design of the liposomes presented a challenge given the requirements of bright ratioable fluorescence, excellent dye encapsulation, minimal interactions with ASL components, rapid osmotic equilibration, and slow solute equilibration. The inclusion of PEG-labeled PC at 8 mol percent conferred a polar exterior surface that has been reported to markedly slow the organ uptake of intravenously injected liposomes and increase circulation time (Allen et al., 1991; Phillips et al., 1999; Working et al., 1999). The inclusion of cholesterol at 30 mol percent decreased liposome fluidity, resulting in decreased incorporation of lipidic constituents from the ASL as well as decreased solute and dye permeabilities. The 400-nm-diam liposome size was optimal to minimize fusion to the cell surface, prolong solute equilibration time (compared with smaller liposomes), and encapsulate sufficient fluorophore to give bright signals in the ASL. Calcein was chosen at the volume-sensitive fluorophore because of its excellent self-quenching properties with minimal spectral shift, pH-insensitivity, and sustained incorporation in liposomes. In addition, the availability of a potent quencher of calcein fluorescence ( $\text{Co}^{2+}$ ; Wallach and Steck, 1963) per-

mitted detection and quenching of small amounts of calcein that may leak from liposomes. Other volume-sensing strategies (chlorocarboxyfluorescein self-quenching, and various fluorophore-quencher pairs) were found to be inferior. Sulforhodamine 101 was chosen as the volume-insensitive reference fluorophore because of its bright red fluorescence, lack of self-quenching, pH independence, and sustained incorporation in liposomes. Sulforhodamine 101 was used previously as a reference dye in measurements of Golgi pH using a liposome fusion method (Seksek et al., 1995). Finally, dye concentrations and osmolality of the liposome preparation were optimized to maximize the sensitivity of the calcein/sulforhodamine 101 fluorescence ratio to solution osmolality.

The findings here of an approximately isosmolar ASL, taken together with water permeability measurements and the computations in Fig. 5, suggest that ASL osmolality differs little from serum osmolality under normal physiological conditions. Maneuvers to increase or decrease ASL osmolality by nebulization of hypertonic or hypotonic solutions are predicted to produce only transient changes in ASL osmolality. However, although the osmotic water permeability of the airway epithelium is fairly high, evaporative water loss by tracheostomy or rapid dry air breathing can substantially increase ASL osmolality. The potential clinical relevance of this observation with respect to cough and reactive airway disease will require further investigation.

The lack of effect of CFTR inhibitors in the cell culture studies, the comparable ASL osmolalities in wild type and CF mice, and the fairly high water permeability of the tracheal epithelium suggest that ASL osmolality is not an important factor in the pathogenesis of CF. These results are in agreement with our recent findings of similar salt concentrations ( $[\text{Na}^+]$  and  $[\text{Cl}^-]$ ) using the same cell culture and mouse systems. However, it is recognized that cell cultures are imperfect models of the airways, and CF mice are imperfect models of human CF because the mice develop little pulmonary disease. Definitive noninvasive measurements in human airways may be required to definitively establish the role of ASL fluid composition in the pathogenesis of CF. Notwithstanding these caveats, investigation of alternative CF pathogenesis mechanisms are warranted, such as abnormalities in gland secretory function.

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