Hypertonic Saline Therapy in Cystic Fibrosis

**EVIDENCE AGAINST THE PROPOSED MECHANISM INVOLVING AQUAPORINS**

Received for publication, May 5, 2006, and in revised form, July 5, 2006 Published, JBC Papers in Press, July 7, 2006, DOI 10.1074/jbc.M604332200

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Recent data indicate the clinical benefit of nebulized hypertonic saline in cystic fibrosis lung disease, with a proposed mechanism involving sustained increase in airway surface liquid volume. To account for the paradoxical observation that amiloride suppresses the beneficial effect of hypertonic saline, it has been previously concluded (Donaldson, S. H., Bennett, W. D., Zeman, K. L., Knowles, M. R., Tarran, R., and Boucher, R. C. (2006) N. Engl. J. Med. 354, 241–250) that amiloride-inhibitable aquaporin (AQP) water channels in airway epithelia modulate airway surface liquid volume. Here, we have characterized water permeability and amiloride effects in well differentiated, primary cultures of human airway epithelial cells, stably transfected Fisher rat thyroid epithelial cells expressing individual airway/lung AQPs, and perfused mouse lung. We found high transepithelial water permeability ($P_w$, 54 ± 5 μm/s) in airway epithelial cells that was weakly temperature-dependent and inhibited by >90% by reduced pH in the basal membrane-facing solution. Reverse transcription-PCR and immunofluorescence suggested the involvement of AQPs 3, 4, and 5 in high airway water permeability. Experiments using several sensitive measurement methods indicated that amiloride does not inhibit water permeability in non-cystic fibrosis (non-CF) or CF airway epithelia, AQP-transfected Fisher rat thyroid cells, or intact lung. Our data provide evidence against the mechanism proposed by Donaldson et al. to account for the effects of amiloride and hypertonic saline in CF lung disease, indicating the need to identify alternate mechanisms.

Cystic fibrosis (CF) is a relatively common hereditary disease in Caucasians caused by mutations in the CF transmembrane conductance regulator (CFTR) chloride channel. Morbidity and mortality in CF result primarily from chronic airway infection, which results in progressive deterioration of lung function.

Two recent clinical studies have demonstrated short and long term benefits of nebulized hypertonic saline in improving lung function in CF (1, 2). Prior clinical studies also support the efficacy of various inhaled hyperosmolar agents in CF (3–6). The recent study by Donaldson et al. (1) concludes that hypertonic saline produces sustained elevation in airway surface liquid (ASL) volume, which improves mucociliary clearance in the airways. However, a paradoxical effect was found when amiloride was administered together with hypertonic saline. Rather than improving lung function because of its inhibitory effect on ENaC and consequent prevention of ASL absorption, amiloride negated the beneficial effect of hypertonic saline. To account for these findings, Donaldson et al. (1) postulated the involvement of airway AQPs in establishing ASL volume (Fig. 1 and reported strong amiloride inhibition of osmotic water permeability in airway epithelial cells. In their model, ENaC hyperactivity dehydrates the ASL in CF airways (Fig. 1, left panel), and hypertonic saline restores ASL volume (middle panel). If amiloride acts only on ENaC, then amiloride is predicted to increase ASL volume (Fig. 1, right, top panel). To account for the clinical data, they hypothesize that amiloride inhibits airway AQPs, which prevents water from entering the airways (Fig. 1, right, bottom panel).

The proposed involvement of AQPs in ASL regulation is surprising, as is the inhibition of AQP water permeability by amiloride. Osmotically induced water transport across airways has been shown to be high and likely AQP-dependent in microperfused small airways (7) and later in cultured human tracheal epithelial cells (8) and in spheroids composed of airway epithelial cell monolayers (9). Despite this, the reduction of airway epithelial water permeability by deletion of the various lung/airway aquaporins (AQPs 1, 3, 4 and 5) in transgenic mice did not affect ASL volume or ionic composition (10). Inhibition of AQP water permeability by amiloride is surprising, because amiloride has no aquagretic effect at concentrations that inhibit ENaC in the renal distal tubule.

Here, we tested the hypothesis of Donaldson et al. (1) by characterizing water permeability and amiloride effects in human bronchial epithelial cell cultures, stably transfected epithelial cells expressing airway/lung AQPs, and intact lung using a variety of biophysical methods to quantify water transport. Water permeabilities of airway cells and intact lung surface
were found to be high and AQP-dependent, although amiloride did not inhibit water permeability nor did it inhibit water permeability of the individual AQPs. These findings have important implications for understanding the benefit of hypertonic saline in CF and for future development of related strategies for the therapy of CF lung disease.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Surface bronchial epithelial cells were obtained from non-CF human lung specimens that were not suitable for lung transplantation. CF bronchial epithelial cells were obtained from patients at the time of transplantation. The cells were dissociated by enzymatic digestion, as described previously (11). Isolated cells were suspended in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium containing 5% fetal calf serum (Hyclone, Logan, UT), gentamicin (50 μg/ml), penicillin (100 units/ml), streptomycin (1 mg/ml), and fungizone (2.5 μg/ml) and seeded as passage 0 (P0) cultures at a density of 10⁶ cells/cm² onto 12-mm Transwell polycarbonate inserts (0.4 μm pore size; Costar, Corning, NY) overlaid with a thin coat (15 μg/cm²) of human placental collagen (Sigma-Aldrich). The next day, the cells were rinsed with PBS, and the medium was replaced with air-liquid interface medium (12) containing apical solution. In other experiments, the osmotic gradient was reversed by the addition of mannitol to the basal rather than the dye-containing apical fluid were collected at specified times. The samples were diluted in 2 ml of PBS, and fluorescence was measured by cuvette fluorimetry (Fluoro Max-3; Horiba, Tokyo, Japan). In some experiments, the pH of either the basal or apical solutions was adjusted to 5.0 using buffers containing 137.6 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 6 mM d-glucose, and 10 mM HEPES (for pH 7.4) or MES (for pH 5.0). In some experiments, HgCl₂ (0.2 or 1 mM) was added to the apical and basolateral solutions 5 min prior to the osmotic gradient.

For computation of transepithelial osmotic water permeability coefficients (Pₛ in cm/s), the time course of fluorescence in fetal calf serum, and propagated cells were resuspended in plating medium and seeded onto cell culture inserts as described above but designated as passage 1 (P1) cultures. By day 15 after seeding, both P0 and P1 cultures formed tight layers with transepithelial resistances of 1–2 kΩ·cm², at which time they were used for experiments. Cultures were confirmed to express CAMP-activated CFTR by measurement of short circuit current, as reported previously (13). Protocols were approved by the University of California, San Francisco Committee on Human Research.

Fisher rat thyroid (FRT) epithelial cells stably transfected with either control plasmid (encoding yellow fluorescent protein) or with plasmids encoding AQP1, AQP3, or AQP4 (14) were grown in F-12/Coombs medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Hyclone), penicillin G (100 units/ml), streptomycin (100 μg/ml), and the appropriate antibiotic selection markers. For dye dilution experiments, FRT cells were plated onto uncoated Transwell inserts and used for flux measurements at resistances of 2–5 kΩ·cm².

**Transepithelial Water Permeability**—Osmotic water permeabilities across human bronchial epithelial (HBE) and FRT cell layers were determined using a dye dilution method, as depicted in Fig. 2A. The dilution of a cell-impermeant, photo-stable, inert dye (Texas Red™-dextran, 10 kDa; Molecular Probes, Eugene, OR) was used as a measure of transcellular osmotic water flux. The basal surface of cells on the porous filter was bathed in 1 ml of isosmolar PBS. The apical surface was bathed in 200 μl of hyperosmolar PBS (PBS + 300 mM D-mannitol) containing 0.25 mg/ml Texas Red-dextran. In some experiments, 100–500 μM amiloride (Sigma-Aldrich), dissolved freshly from powder, was added to the apical buffer, as done by Donaldson et al. (1), or to both the apical and basal bathing buffers. Cultures were placed in a 5% CO₂ tissue culture incubator (at 27 or 37°C), and 5 μl samples of dye-containing apical fluid were collected at specified times. The samples were diluted in 2 ml of PBS, and fluorescence was measured by cuvette fluorimetry (Fluoro Max-3; Horiba, Tokyo, Japan). In some experiments, the osmotic gradient was reversed by the addition of mannnitol to the basal rather than the dye-containing apical solution. In other experiments, the pH of either the basal or apical solutions was adjusted to 5.0 using buffers containing 137.6 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 6 mM D-glucose, and 10 mM HEPES (for pH 7.4) or MES (for pH 5.0). In some experiments, HgCl₂ (0.2 or 1 mM) was added to the apical and basolateral solutions 5 min prior to the osmotic gradient.

For computation of transepithelial osmotic water permeability coefficients (Pₛ in cm/s), the time course of fluorescence in
response to solution osmolarity changes, \( F(t) \), was fitted to a single exponential time constant, \( F(t) = F_0 + A e^{-t/\tau} \), where \( F_0 \) is initial fluorescence, \( A \) is amplitude, and \( \tau \) is the exponential time constant. Because of dye binding to cells and/or the support flux, data used for exponential regression was collected at 5 min and later after establishing the osmotic gradient. Water flux was computed as the rate of dye dilution by \( dV(0)/dt = V_o/\tau \), which follows from the relation: \( F(t) = F_o = V_o/\tau + V(t) \). \( V_o \) is the initial volume of dye-containing apical buffer (0.2 cm\(^3\)), and \( V(t) \) is the apical solution volume at time \( t \).

From these equations, \( P_f \) was computed as \( dV(0)/dt = P_f \nu_o endorse (\Phi_1 - \Phi_2) \), where \( S \) is the tissue surface area assuming a smooth surface (1.13 cm\(^2\)), \( \nu_o \) is the partial molar volume of water (18 cm\(^3\)/mol), and (\( \Phi_1 - \Phi_2 \)) is the transepithelial osmotic gradient (\( 3 \times 10^{-4} \) mol/cm\(^2\)).

**Plasma Membrane Water Permeability**—Plasma membrane osmotic water permeability was measured using a calcium-quenching method (shown in Fig. 3A) that has been applied previously in vitro and in vivo cell systems (15, 16). Cells were loaded with calcein by incubation with PBS containing 10 \( \mu \)M calcein-AM (Molecular Probes) for 30 min at 37°C. HBE cultures grown on collagen-coated supports were studied by cutting the flat supports from the plastic inserts and mounting them in a custom-built perfusion chamber with support bottoms pressed against a coverslip and apical cell surfaces exposed to the perfusate. FRT cells grown on glass coverslips were inserted in a similar perfusion chamber but with cells facing upwards. Solutions were exchanged between PBS (290 mOsm) and hyperosmolar PBS (590 mOsm, PBS with added d-mannitol) using a gravity pinch valve system (ALA Scientific Instruments, Westbury, NY). Perfusates contained either \( \text{Me}_2\text{SO} \) vehicle (0.1%) or amiloride (500 \( \mu \)M) added from a 1000 \( \times \) \( \text{Me}_2\text{SO} \) stock solution. Fluorescence was excited using an X-cite 120 mercury lamp (EXFO Life Sciences, Ontario, Canada) and focused through an inverted epifluorescence microscope through a long working distance 25 \( \times \) air objective (numerical aperture 0.35, Leitz). Excitation and emission light was filtered using a custom cube (Chroma, Rockingham, VT), and fluorescence was collected using a 14-dynode photomultiplier and then amplified, digitized, and recorded using custom software (written in LabView, National Instruments, Austin, TX).

To compute plasma membrane \( P_f \), relative fluorescence \( (F(t)/F_o) \) was fitted to a single exponential function (as above) for FRT cell studies or a biexponential function \( (F(t)/F_o) = A e^{-t/\tau_1} + B e^{-t/\tau_2} + C \) for HBE studies. Plasma membrane \( P_f \) was computed as described above, where surface area \( S \) was taken as the apical surface area of the cell monolayer, assuming a flat, smooth surface. The time-varying cell layer height, \( h(t) \), was related to normalized amplitudes according to the predicted 50% final decrease from initial height \( (h_0) \) under hypertonic conditions. For FRT and HBE cells, \( h_0 \) was taken as 5 and 20 \( \mu \)M, respectively. \( P_f \) was then computed from \( dh(0)/dt = P_f \nu_o endorse (\Phi_1 - \Phi_2) \).

**Airspace-Capillary Water Permeability in Intact Lung**—Wild-type and AQP5-deficient mice in a CD1 genetic background (age 8–14 weeks) (17) were sacrificed using an overdose of 2,2,2-tribromoethanol (avertin, Sigma-Aldrich) intraperitoneally. Protocols were approved by the University of California, San Francisco Committee on Animal Research. The trachea was transected and cannulated, the pulmonary artery was cannulated, and the heart and lungs were moved en bloc to a perfusion chamber as described previously (18). After ensuring that no air leaks were present, 0.5 ml of fluorescein isothiocyanate-dextran solution (10 kDa, 0.5 mg/ml in PBS; Sigma-Aldrich), with or without freshly dissolved amiloride (500 \( \mu \)M), was instilled into the tracheal catheter. The pulmonary artery was perfused at constant pressure (~60 cm H\(_2\)O) with PBS, with or without 500 \( \mu \)M amiloride, for at least 5 min before measurements. The pleural surface was washed continuously with PBS, and perfusate and wash effluent were continuously withdrawn from the chamber by suction (perfusion flow 5 ml/min). The time course of fluorescence intensity from a 3–5-mm spot on the lung pleural surface, in a similar location for all samples, was continuously monitored while perfusate fluid was exchanged between isosmolar and hyperosmolar PBS (PBS + 300 \( \mu \)M d-mannitol) using the microscope setup, detection system, and gravity pinch valve system described above.

**Histology and Immunocytochemistry**—Paraffin sections of P0 HBE cell culture inserts and fixed human trachea were prepared. For paraffin sections, sections were fixed for at least 30 min in buffered formalin (10%) and stored in 0.1 M phosphate buffer (pH 7.4). Standard tissue dehydration and paraffin infiltration was performed in a Tissue-Tek VIP processor (Sakura Finetek, Torrance, CA). Sections were cut at 4 \( \mu \)M on a rotary microtome and stained with hematoxylin and eosin. Specimens were viewed and photographed on an Olympus light microscope (Olympus America, Inc., Melville, NY) equipped with a digital imaging system (QImaging, Burnaby, Canada). Immunostaining was done by standard procedures using polyclonal antibodies for AQP3, AQP4, and AQP5 (Chemicon, Temecula, CA) incubated for 2 h and Cy3-conjugated goat anti-mouse IgG (1:200; Sigma-Aldrich) incubated for 30 min.

**Reverse Transcription (RT)-PCR**—Total RNA from HBE cells scraped freshly from P0 culture inserts was isolated by homogenization in TRIzol reagent (Invitrogen), and mRNA was extracted using the Oligotex mRNA midi kit (Qiagen, Valencia, CA). cDNA was reverse transcribed from mRNA with oligo(dT) (SuperScript II preamplification kit, Invitrogen). Primers were designed to amplify 300–350 base pair fragments of cDNAs encoding human \( \beta \)-actin (sense, 5'-GAGTGGTCTCTGTGGCGATCC-3'; antisense, 5'-CATTTTGGC- GTGGACAGGTGAC-3'), AQP1 (sense, 5'-GCCATCGGC- CTCCTCTGAGGC-3'; antisense, 5'-CTATTTGGGCTCT- ATCTGAC-3'), AQP2 (sense, 5'-ACCTCTCTGGTGGAC- TATTACA-3'; antisense, 5'-TCAGGGCTTGTGGTACCCCG- TGG-3'), AQP3 (sense, 5'-CTGTGGTGTCCTGGTGC- C-3'; antisense, 5'-CTGCTCCTGGTGCCTACAT-3'), AQP4 (sense, 5'-GGACCTGGTGAATCTCATGGAC-3'; antisense, 5'-CATACCTCTCCGATGTTGC-3'), AQP5 (sense, 5'-CTGTCCATTGGCTGCTGCT-3'; antisense, 5'-GCGGGTGCTCAGCTCCATG-3'), AQP6 (sense, 5'-CTGGGCCACCTCCTCGAT-3'; antisense, 5'-TCACACCCTCCTCAGTCCGAC-3'), AQP7 (sense, 5'-CAGGTCTTCA- CAGTGGGAG-3'; antisense, 5'-CTCTAGGGCCATGG-
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ATTGCTG3′), AQP8 (sense, 5′-GTGGCAGATGCATC-CTGACG-3′; antisense, 5′-TTCAGATGAGCGGTGGT- TTC-3′), and AQP9 (sense, 5′-GACTCCAGAATACTGGG- AGCC-3′; antisense, 5′-TTGTCCTCAGATTGTCTGCG-3′). RT-PCR (for stained agarose gels) was performed with the TaqDNA polymerase kit (Invitrogen), and PCR products were electrophoresed on a 1.2% agarose gel.

Fluorescence-based real-time RT-PCR was done to compare relative AQP3, AQP4, and AQP5 mRNA expression in non-CF versus CF cells using the LightCycler™ and with the LightCycler FastStart DNA MasterPLUS SYBR Green I kit (Roche Diagnostics) according to the manufacturer’s instructions. Surface epithelial cells were dissociated from human bronchi, quick-frozen, and stored at −80 °C until thawed for RNA isolation. PCR primers were as described above. Results were reported as a normalized, calibrated ratio with all samples normalized to β-actin. Concentration ratios for each CF and non-CF sample were calibrated to calibrator samples (pooled cDNA from non-CF subjects), with the results reported as a normalized ratio with the calibrator sample as the denominator as follows: relative mRNA level = ratio of sample (target/reference)/ratio of calibrator (target/reference).

RESULTS

Water transport in airway surface epithelium was studied using well differentiated HBE cell cultures. Transepithelial osmotic water permeability was measured using a dye dilution method that assays water flux across tight epithelia cultured on porous filters (Fig. 2A). The fluorescence of an apical solution volume marker provided a quantitative readout of osmotically driven water transport across the cell layer. An induced osmotic gradient caused transepithelial water movement from which osmotic water permeability (Pf) was deduced. The P0 non-CF cultures were first characterized. Fig. 2B (top) shows fluorescence dilution at 37 °C, in which a 300 mmHg gradient of D-mannitol induced osmotic water flux into the apical, dye-containing solution. Data in the absence versus the presence of amiloride (500 μM) were compared. Fig. 2C summarizes transepithelial Pf values. There was no significant difference in Pf upon exposure of P0 HBE cultures to high dose amiloride (control versus amiloride, 54 ± 5 versus 52 ± 7 μm/s). Lower concentrations of amiloride (100 and 250 μM) also did not change Pf (not shown).

When the direction of the osmotic gradient was reversed by the addition of mannitol to the basal bathing solution, the fluorescent volume marker became concentrated over time (Fig. 2B, middle). The deduced transepithelial Pf (46 ± 15 μm/s) did not differ significantly from that in control studies (mannitol in apical solution), indicating symmetrical water transport. To investigate the involvement of AQPs in HBE water permeability, experiments were done at reduced temperature (27 °C) and with reduced pH in the apical or basal bathing solutions (Fig. 2C, bottom). Pf at 27 °C (47 ± 9 μm/s) was similar to that at 37 °C, indicating a low Arrhenius activation energy, as expected for AQP-facilitated water transport. Reducing apical solution pH to 5 did not change Pf significantly, whereas a basolateral solution pH of 5 decreased Pf by >90%, providing functional evidence for the involvement of basolateral membrane AQP3 in water permeability; the water permeability of AQP3 is inhibited at low pH, whereas that of other AQPs is not (19).

Non-CF and CF P1 cell cultures were also studied to mimic the culture conditions employed by Donaldson et al. (1). As summarized in Fig. 2D, P1 cultures had −50% lower water permeability than P0 cultures but without significant differences comparing non-CF versus CF cultures or control cultures versus cultures exposed apically to 500 μM amiloride (or to 100 μM amiloride, not shown). Finally, as an additional attempt to look for amiloride-sensitive water transport, water permeability was
AQP expression was characterized in P0 HBE cells. Hema
toxylin and eosin-stained transverse paraffin sections con
tirmed the differentiation of 2-week-old cultures into a
−20-μm thick pseudostratified epithelium with numerous cilia (Fig. 4A). Cultures were scraped for RT-PCR analysis, which
detected mRNA for several AQPs (Fig. 4B). Transcripts of
AQP3, AQP4, and AQP5 were found in highest abundance,
consistent with previous studies (see “Discussion”). Lesser but
non-zero expression of AQP1, AQP6, and AQP7 mRNA was
detected. Quantitative real-time RT-PCR was done to compare
expression of the principal AQPs in P0 HBE cultures from
non-CF versus CF airways. As summarized in Fig. 4C, no signif-
ificant differences were found in AQP transcript expression in
non-CF versus CF cells. Immunofluorescence done on paraffin
sections of HBE cultures and intact human bronchus demon-
strated AQP3 and AQP4 protein expression on basolateral
membranes, whereas AQP5 protein expression was not
detected. Positive control staining of kidney for AQP3 and
AQP4 is shown and of salivary gland for AQP5.

We next investigated the possible effects of amiloride on
the major lung AQPs, including AQP1 (expressed in micro-
vascular endothelia), using AQP-transfected epithelial cells
and intact mouse lung. Transepithelial and plasma mem-
brane water permeabilities were measured in FRT cells
expressing either yellow fluorescent protein (control),
AQP1, AQP3, or AQP4. Transepithelial water permeability
of FRT cell lines grown on Transwell supports was measured
after the cultures formed electrically tight monolayers.
Water permeabilities of AQP-expressing FRT cells were
greater than that in control cells and not sensitive to amilo-
ride (Fig. 5A, top). Fig. 5A (bottom) summarizes transepithe-
lial \( P_f \) values. Plasma membrane water permeability was mea-
sured on the FRT cells, with calcein fluorescence curves fitted
to single exponential functions (representative traces for FRT-
AQP3 and FRT-AQP4 cells shown in Fig. 5B, top). In agreement
with the measurements of transepithelial water permeability,
plasma membrane \( P_f \) was increased with AQP expression and
not sensitive to amiloride (Fig. 5B, bottom).

Last, the effect of amiloride was investigated in intact, per-
fused mouse lung using a pleural surface method to measure
airspace-capillary osmotic water permeability (18). The air-
space compartment was filled with isotonic fluid containing
fluorescein isothiocyanate-dextran as a volume marker. The
kinetics of pleural surface fluorescein isothiocyanate-dextran
fluorescence was measured in response to changes in osmolal-
ity of the pulmonary artery perfusate. There was rapid osmotic
equilibration across the airspace-capillary barrier, which was
not affected by the inclusion of 500 μM amiloride in the airway
instillate and pulmonary artery perfusate (Fig. 6A, top). In
agreement with previous results (20), osmotic equilibration was
~10-fold slowed in lungs of AQP5-deficient mice (Fig. 6A, bot-
ttom), indicating the involvement of AQP5 in airspace-capillary

measured in P0 non-CF cultures in the presence of both apically
and basolaterally added amiloride (500 μM). As seen from the
raw fluorescence data in Fig. 2E, osmotic water flux was not
inhibited by high-dose amiloride, indicating the lack of amilo-
ride inhibition of apical or basolateral AQPs.

Plasma membrane water permeability in P0 HBE cultures
was determined using a calcein-quenching method, which is
based on rapid changes in cytoplasmic calcein fluorescence in
response to changes in the concentration of cytoplasmic ani-
onic proteins and hence to changes in cell volume (Fig. 3
B). The kinetics of pleural surface fluorescein isothiocyanate-dextran
fluorescence was measured in response to changes in osmolal-
ity of the pulmonary artery perfusate. There was rapid osmotic
equilibration across the airspace-capillary barrier, which was
not affected by the inclusion of 500 μM amiloride in the airway
instillate and pulmonary artery perfusate (Fig. 6A, top). In
agreement with previous results (20), osmotic equilibration was
~10-fold slowed in lungs of AQP5-deficient mice (Fig. 6A, bot-
ttom), indicating the involvement of AQP5 in airspace-capillary

geometry of bronchial epithelia. \( P_f \) was quantified from the ini-
tial kinetics of fluorescence change in response to osmotic gra-
dients as described under “Experimental Procedures.” As sum-
marized in Fig. 3C, the plasma membrane \( P_f \) was not sig-
nificantly affected by inclusion of 500 μM amiloride in the

FIGURE 3. Plasma membrane water permeability of human bronchial cell
cultures. Water permeability measured by a calcein fluorescence-quenching
method. A, schematic of cell volume-dependent calcein quenching, where
apical plasma membrane exposure to hypertonic buffer induces cell shir-
kling, reducing cytoplasmic calcein fluorescence because of quenching by ani-
onic cytoplasmic proteins. B, representative time courses of calcein fluores-
cence in HBE cells in response to hyperosmolar challenge followed by return
to isosmolar solution, in the absence (top) or presence (bottom) of 500 μM
amiloride. Double exponential fits used for \( P_f \) determination are overlaid (see
“Experimental Procedures”). C, summary of calculated \( P_f \) (3–6 curves aver-
ged for individual cultures, three cultures/condition ± S.E.). Differences
were not significant.
The primary purpose of this study was to investigate a proposed AQD-dependent mechanism to account for the clinical effects of nebulized hypertonic saline (HS) and amiloride on lung function in CF. The validity of the proposed mechanism of clinical benefit involving amiloride-sensitive airway AQPs has important implications regarding the use of HS versus non-salt hyperosmolar agents in CF therapy, as well as in the proposed use of amiloride-type ENaC inhibitors in preventing ASL dehydration.

HS is being used increasingly as a chronic therapy in CF, particularly following the recent publication of two clinical trials in the New England Journal of Medicine demonstrating clinical benefit (1, 2). The larger of the two studies reported that 4 ml of 7% HS nebulized twice daily for 48 weeks decreased exacerbation rate by 56% in CF subjects (2). Exacerbations were defined by the need for intravenous antibiotics or using a symptom score involving increased sputum production, lethargy, dyspnea, and fever. In previous smaller studies of CF subjects, a single nebulized dose of 6–7% HS increased mucociliary clearance at 60 and 90 min, as measured by radionucleotide imaging (3, 5). Increased mucociliary clearance was dose-dependent up to 12% HS in 10 adults treated with a single dose and tested at 90 min (4). In a 2-week study in 52 CF subjects, 10 ml of 6% HS improved lung function as measured by a 12% increase in forced expiratory volume in 1 s (FEV1) (6). HS has also been shown to increase mucociliary clearance in healthy subjects and in subjects with asthma or chronic obstructive lung disease (21, 22).

Various non-salt hyperosmolar agents have been posited to improve mucus clearance in CF and other chronic inflammatory lung diseases. The best-studied non-salt hyperosmolar agent is mannitol. In 12 CF subjects, single dose administration of 300 mg of mannitol as a dry powder improved bronchial mucus clearance at 60 min, although there was a small decline in FEV1 immediately after...
inhalation that was unresponsive to bronchodilator premedication (5). The same dose of mannitol improved mucus clearance 2-fold in healthy, asthmatic, and bronchiectatic subjects (21, 23). A 12-day trial of 400 mg of mannitol daily in nine non-CF bronchiectatic subjects gave no change in FEV1 but improved various sputum characteristics, such as surface tension and sputinability (24). Another inhaled hyperosmolar agent, xylitol, reduced ASL salt concentration in CF and non-CF airway epithelia in vitro (25). Pretreatment of human airway explants for 4 h with 60–80 mg/ml xylitol reduced binding of a Burkholderia cepacia isolate (26).

As part of the present study, we characterized AQP expression and function in water transport across human bronchial epithelial cells. As discussed in the Introduction, a central prediction of the mechanism proposed by Donaldson et al. (1) is the inhibition of AQP water permeability by amiloride. To support their mechanism, the authors measured transepithelial flux across HBE cultures in response to an apically directed mannitol gradient, as we have done here (Fig. 2A), with two differences. First, in their study, the concentration of Texas Red-dextran was measured in the basolateral (rather than the apical) solution. Second, in their study, fluorescence was measured by confocal microscopy without any buffer sampling.

FIGURE 5. Transepithelial and membrane water permeabilities in AQP-transfected FRT cell cultures. A, transepithelial osmotic water permeability of transfected FRT cells measured by dye-dilution. Top, averaged time courses of osmotic water movement in FRT cell cultures transfected with yellow fluorescent protein (YFP) (circles), AQP1 (squares), AQP3 (diamonds), and AQP4 (triangles), with apical solution fluorescence normalized as F/F0. Control cultures (open symbols) were compared with cultures exposed to 500 μM amiloride at their apical surface (closed symbols). Measurements performed at 37 °C (three cultures/group ± S.E.). Single exponential fits are shown as solid lines. Bottom, summary of P1 values. Differences were not significant. B, plasma membrane osmotic water permeabilities of yellow fluorescent protein- and AQP-transfected FRT cells measured by calcine quenching. Top, representative time courses of calcine fluorescence in cultures of AQP3 (top curve) and AQP4 (bottom curve)-transfected cells in response to hyperosmolar challenge and return to isosmolar solution, in the absence of amiloride. Bottom, summary of P1 (4–8 curves averaged from single cultures, three sets of cultures/condition ± S.E.). Differences were not significant.

They detected membrane transport rates as changes in cell height over time, with cytoplasmic calcine fluorescence imaged using reconstructed x–z confocal stacks. From both approaches, they reported a >70% reduction in osmotic water transport by 100–400 μM amiloride in non-CF and CF HBE cultures. Amiloride also prevented the transient increase in ASL volume upon acute addition of perfluorocarbon-dispersed NaCl to the mucosal surface of epithelial cultures, although this approach does not provide a direct measure of osmotic water permeability and was thus not replicated here. HgCl2, a toxic and nonspecific inhibitor of AQPs, dramatically inhibited this ASL volume response, although this finding appears at odds with their prior report of a much milder (<30%) reduction of transepithelial and membrane water permeability by apically added HgCl2 (8). Based on these data, the authors proposed that inhibition of airway AQPs by amiloride accounted for the clinical effect and supported the hypothesis of ASL dehydration in CF.

Here, using essentially the same airway epithelial cell culture model used by Donaldson et al., we found no effect of amiloride on transepithelial or plasma membrane osmotic water permeability. Our fluorescence-based methods were capable of detecting small water permeability differences of <10%. Also, we found no effect of amiloride on the water permeability of individual airway/lung AQPs in transfected epithelial cells or in AQP5-dependent airspace-capillary water permeability in intact perfused mouse lung. We conclude that amiloride does not inhibit osmotically driven water transport in the airways or lung, indicating the need to identify other mechanism(s) to explain the paradoxical clinical observations reported by Donaldson et al. The discrepancy between our findings and those of Donaldson et al. may be related to differences in measurement techniques, as discussed above. The techniques of Donaldson et al. (1) were first employed by Matsui et al. (8) in characterizing HBE water permeabilities. This earlier study reported in both non-CF and CF P1 cultures a plasma membrane P1 (~90 μm/s) ~2-fold less than transepithelial P1 (~160 μm/s), which is a physical impossibility for a tight epithelium, because the apical and basolateral membranes are barriers in series. Here, our results for P0 cultures are very different, a higher membrane P1 (~200 μm/s), which is ~4-fold greater than transepithelial P1 (~50 μm/s). For non-CF and CF P1 cultures, we found a lower transepithelial
cells in human large airways (32, 33). In well differentiated primary cultures of human airway epithelial cells and in fixed human bronchi, we found here the expression of AQP3 and AQP4 in the basolateral membranes of airway epithelia but did not detect AQP5 protein expression. However, the lack of AQP5 immunostaining probably reflects a lack of antibody specificity rather than the absence of functional protein, given the demonstrated presence of AQP5 in nasal and bronchial epithelial cells in previous studies (33).

Prior functional studies have suggested the involvement of AQPs in airway water permeability. Our initial measurements in microperfused small airways from guinea pigs showed high transepithelial water permeability ($P_f \approx 50 \mu m/s$) that was weakly temperature-sensitive and was mercury-insensitive, suggesting the involvement of AQP4 (7). Recent data from spheroid explants of human nasal polyps provided evidence for AQP5 function in airway epithelia. In these large, spherical airway cell monolayers, with the ciliated apical membrane facing outward, Pederson et al. (9) reported high membrane $P_f (\approx 150 \mu m/s)$ that was sensitive to brief exposure to low concentration mercury. Here, our high $P_f$ values for HBE cultures and the weak temperature dependence of permeability suggest AQPs-mediated water movement (Fig. 2). Additionally, the reduced transepithelial osmotic water permeability under low pH in the basal (but not apical) membrane-facing solution provides indirect evidence for the function of AQP3 (the only pH-regulated airway AQP) in HBE basolateral membranes (19). The addition of HgCl$_2$ to the cultures used here resulted in marked leakiness with reduced transepithelial resistance, precluding conclusions about HgCl$_2$ effects. CF cultures had similar water permeability to non-CF cultures, consistent with our RT-PCR data (Fig. 3C) and with previous reports (8, 9).

On theoretical grounds the functioning of airway AQPs is not expected to be a significant determinant of ASL volume, composition, or other properties. Because the lipid bilayer in plasma membranes has substantial water permeability, the presence of AQPs generally increases plasma membrane water permeability by only 5–10-fold, which is important for rapid fluid movement, such as in kidney or salivary gland, but not for the many orders of magnitude slower fluid movement in the lung (reviewed in Ref. 34). Indeed, despite 10-fold slowed osmotic water permeability in mouse lung by the deletion of AQP1 and AQP5, isosmolar fluid absorption was unaffected (20, 35). Also, we reported previously that deletion of each of the airway/lung aquaporins (AQP1, AQP3, AQP4, and AQP5), individually and in combinations, did not affect ASL volume or ionic composition and had only a minimal effect of airway hydration at maximal respiratory rates (10).

The discussion above indicates the difficulty in ascribing aquaporin function, and increased ASL volume in general, to account for the effects of nebulized HS. Another potential problem with the hypothesis of increased ASL volume is that the excess fluid added to the intact airways/lung is likely to dissipate over minutes or tens of minutes rather than produce a sustained increased in ASL volume over hours between hypertonic saline treatments. Because of the large surface-to-volume ratio of the alveoli and airways and their high water permeability, added hypertonic fluid becomes nearly isotonic within 1
min due to osmotic water efflux into the airspaces. We showed rapid osmotic equilibration initially in sheep lung (36) and later in other mammals (18), including mouse as seen in Fig. 6. Because of rapid isosmolar fluid absorption in mammalian lung, including human lung (reviewed in Ref. 37), the ~40 ml of isosmolar fluid produced by nebulizing 5 ml of 7% HS is likely to be absorbed in <15–30 min. However, the exact clearance rate of HS would depend on its distribution in small versus large airways following nebulization. Rapid isosmolar fluid absorption has also been shown in the CF lung despite absent CFTR, because basal cAMP-independent lung fluid absorption does not involve CFTR (38). Thus, it is difficult to reconcile the clinical benefit of HS with the expected transient increase in ASL volume; however, the exact location of excess fluid, in terms of airways versus alveoli and periciliary versus mucus fluid, is not known. The increased ASL volume mechanism would also predict a better outcome for inhaling non-absorbed solutes, such as mannitol or xylitol, which from clinical studies is not the case.

Alternative mechanisms have been proposed to account for the apparent clinical benefit of inhaled hyperosmolar agents. One mechanism involves the direct effects of NaCl and mannitol on mucus rheology (5). HS has been proposed to reduce the apparent clinical benefit of inhaled hyperosmolar agents.

Acknowledgments—We thank Lorna Zlock and Jean Davidson for preparing cultures of human airway epithelia, Dr. Michael Matthey for assistance in the acquisition of lung specimens, Dr. Dan Zhao for help with RT-PCR analysis, Dr. Yaunlin Song for technical advice on mouse lung perfusion, and Liman Qian for mouse breeding and genotype analysis.

REFERENCES

1. Donaldson, S. H., Bennett, W. D., Zeman, K. L., Knowles, M. R., Tarran, R., and Boucher, R. C. (2006) *N. Engl. J. Med.* 354, 241–250

2. Elkins, M. R., Robinson, M., Rose, B. R., Harbour, C., Moriarty, C. P., Marks, G. B., Belousova, E. G., Xuan, W., Bye, P. T., and the National Hypertonic Saline in Cystic Fibrosis (NHSCF) Study Group (2006) *N. Engl. J. Med.* 354, 229–240

3. Robinson, M., Regnis, J. A., Bailey, D. L., King, M., Bautovich, G. J., and Bye, P. T. (1996) *Am. J. Respir. Crit. Care Med.* 153, 1503–1509

4. Robinson, M., Hemming, A. L., Regnis, J. A., Wong, A. G., Bailey, D. L., Bautovich, G. J., King, M., and Bye, P. T. (1997) *Thorax* 52, 900–903

5. Robinson, M., Daviskas, E., Eberl, S., Baker, J., Chan, H. K., Anderson, S. D., and Bye, P. T. (1999) *Eur. Respir. J.* 14, 678–685

6. Eng, P. A., Morton, J., Douglass, J. A., Riedler, J., Wilson, J., and Robertson, C. F. (1996) *Pediatr. Pulmonol.* 21, 77–83

7. Folkesson, H., Matthay, M., Frigeri, A., and Verkman, A. S. (1996) *J. Clin. Investig.* 97, 664–671

8. Matsui, H., Davis, C. W., Tarran, R., and Boucher, R. C. (2000) *J. Clin. Investig.* 105, 1419–1427

9. Pedersen, P. S., Prockop, K., Larsen, P. L., Holstein-Rathlou, N. H., and Frederiksen, O. (2005) *Pfluegers Arch.* 451, 464–473

10. Song, Y., Jayaraman, S., Yang, B., Matthey, M. A., and Verkman, A. S. (2001) *J. Gen. Physiol.* 117, 573–582

11. Yamaya, M., Finkbeiner, W. E., Chun, S. Y., and Widdicombe, J. H. (1992) *Am. J. Physiol.* 262, L713–L724

12. Fulcher, M. L., Gabriel, S., Burns, K. A., Yankaskas, J. R., and Randell, S. H. (2005) *Methods Mol. Med.* 107, 183–206

13. Pedemonte, N., Lukacs, G. L., Du, K., Caci, E., Zegarra-Moran, O., Galietta, L. J., and Verkman, A. S. (2005) *J. Clin. Investig.* 115, 2564–2571

14. Saadoun, S., Papadopoulos, M. C., Hara-Chikuma, M., and Verkman, A. S. (2005) *Nature* 434, 786–792

15. Solenov, E., Watanabe, H., Manley, G. T., and Verkman, A. S. (2004) *Am. J. Physiol.* 286, C426–C432

16. Levin, M. H., and Verkman, A. S. (2004) *Invest. Ophthalmol. Vis. Sci.* 45, 4423–4432

17. Ma, T., Song, Y., Gillespie, A., Carlson, E. J., Epstein, C. J., and Verkman, A. S. (1999) *J. Biol. Chem.* 274, 20071–20074

18. Carter, E. P., Matthey, M. A., Farinas, J., and Verkman, A. S. (1996) *J. Gen. Physiol.* 108, 133–142

19. Zeuthen, T., and Klairke, D. A. (1999) *J. Biol. Chem.* 274, 21631–21636

20. Ma, T., Fukuda, N., Song, Y., Matthey, M. A., and Verkman, A. S. (2000) *J. Clin. Investig.* 105, 93–100

21. Daviskas, E., Anderson, S. D., Gonda, I., Eberl, S., Miekle, S., Seale, J. P., and Bautovich, G. (1996) *Eur. Respir. J.* 9, 725–732

22. Pavia, D., Thomson, M. L., and Clarke, S. W. (1978) *Am. Rev. Respir. Dis.* 117, 199–203

23. Daviskas, E., Anderson, S. D., Brannan, J. D., Chan, H. K., Eberl, S., and Bautovich, G. (1997) *Eur. Respir. J.* 10, 2449–2454

24. Daviskas, E., Anderson, S. D., Gomes, K., Briffa, P., Cochrane, B., Chan, H. K., Young, I. H., and Rubin, B. K. (2005) *Respirology* 10, 46–56

25. Zahnier, J., Seller, M. P., Laanspach, I. L., Karp, P. H., Kearney, W. R., Look, D. C., Smith, J. I., and Welsh, M. J. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 11614–11619

26. Sajjan, U., Moreira, J., Liu, M., Humar, A., Chaparro, C., Forstner, J., and Keshavjee, S. (2004) *J. Heart Lung Transplant* 23, 1382–1391

27. Sood, N., Bennett, W. D., Zeman, K., Brown, J., Foy, C., and Boucher R. C. (2003) *Am. J. Respir. Crit. Care Med.* 167, 158–163

28. App, F. M., King, M., Hellesrieder, R., Kohler, D., and Mattys, H. (1990) *Am. Rev. Respir. Dis.* 141, 605–612

29. Hirsch, A. J., Sabater, J., Zamurs, A., Smith, R. T., Paradiso, A. M., Hopkins, S., Abraham, W. M., and Boucher, R. C. (2004) *J. Pharmacol. Exp. Ther.* 311, 929–938

30. Borok, Z., and Verkman, A. S. (2002) *J. Appl. Physiol.* 93, 2199–2206

31. Hasegawa, H., Lian, S. C., Finkbeiner, W. E., and Verkman, A. S. (1994) *Am. J. Physiol.* 266, C893–C903

32. Nielsen, S., King, L. S., Christiansen, B. M., and Agre, P. (1997) *Am. J. Physiol.* 273, C1541–C1548

33. Kreda, S. M., Gynn, M. C., Fenstermacher, D. A., Boucher, R. C., and Gabriel, S. E. (2001) *Am. J. Respir. Cell Mol. Biol.* 24, 224–234

34. Verkman, A. S. (2005) *J. Cell Biol.* 118, 3225–3232
35. Bai, C., Fukuda, N., Song, Y., Ma, T., Matthay, M. A., and Verkman, A. S. (1999) J. Clin. Investig. 103, 555–561
36. Folkesson, H. G., Matthay, M. A., Hasegawa, H., Kheradmand, F., and Verkman, A. S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4970–4974
37. Matthay, M. A., Folkesson, H., and Verkman, A. S. (1996) Am. J. Physiol. 270, L487–L503
38. Fang, X., Fukuda, N., Barbry, P., Sartori, C., Verkman, A. S., and Matthay, M. A. (2002) J. Gen. Physiol. 119, 199–207
39. King, M., Dasgupta, B., Tomkiewicz, R. P., and Brown, N. E. (1997) Am. J. Respir. Crit. Care Med. 156, 173–177
40. Wills, P. J., Hall, R. L., Chan, W., and Cole, P. J. (1997) J. Clin. Investig. 99, 9–13
41. Feng, W., Garrett, H., Speert, D. P., and King, M. (1998) Am. J. Respir. Crit. Care Med. 157, 710–714
42. Durairaj, L., Launspach, J., Watt, J. L., Businga, T. R., Kline, J. N., Thorne, P. S., and Zabner, J. (2004) Respir. Res. 5, 13