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Research Article

Previous studies have suggested that alveolar Na,K-ATPases play an important role in active Na+ transport and lung edema clearance. We reasoned that overexpression of Na,K-ATPase subunit genes could increase Na,K-ATPase function in lung epithelial cells and edema clearance in rat lungs. To test this hypothesis we produced replication deficient human type 5 adenoviruses containing cDNAs for the rat alpha1 and beta1 Na,K-ATPase subunits (adMRCMValpha1 and adMRCMVbeta1, respectively). As compared to controls, adMRCMVbeta1 increased beta1 subunit expression and Na,K-ATPase function by 2. 5-fold in alveolar type 2 epithelial cells and rat airway epithelial cell monolayers. No change in Na,K-ATPase function was noted after infection with adMRCMValpha1. Rat lungs infected with adMRCMVbeta1, but not adMRCMValpha1, had increased beta1 protein levels and lung liquid clearance 7 d after tracheal instillation. Alveolar epithelial permeability to Na+ and mannitol was mildly increased in animals infected with adMRCMVbeta1 and a similar Escherichia coli lacZ-expressing virus. Our data shows, for the first time, that transfer of the beta1 Na,K-ATPase subunit gene augments Na,K-ATPase function in epithelial cells and liquid clearance in rat lungs. Conceivably, overexpression of Na,K-ATPases could be used as a strategy to augment lung liquid clearance in patients with pulmonary edema.

Augmentation of Lung Liquid Clearance via Adenovirus-mediated Transfer of a Na,K-ATPase β_1 Subunit Gene

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Abstract

Previous studies have suggested that alveolar Na,K-ATPases play an important role in active Na^+ transport and lung edema clearance. We reasoned that overexpression of Na,K-ATPase subunit genes could increase Na,K-ATPase function in lung epithelial cells and edema clearance in rat lungs. To test this hypothesis we produced replication deficient human type 5 adenoviruses containing cDNAs for the rat α_1 and β_1 Na,K-ATPase subunits (adMRCMVα_1 and adMRCMVβ_1, respectively). As compared to controls, adMRCMVβ_1 increased β_1 subunit expression and Na,K-ATPase function by 2.5-fold in alveolar type 2 epithelial cells and rat airway epithelial cell monolayers. No change in Na,K-ATPase function was noted after infection with adMRCMVα_1. Rat lungs infected with adMRCMVβ_1, but not adMRCMVα_1, had increased β_1 protein levels and lung liquid clearance 7 d after tracheal instillation. Alveolar epithelial permeability to Na^+ and mannitol was mildly increased in animals infected with adMRCMVβ_1 and a similar *Escherichia coli lacZ*-expressing virus. Our data shows, for the first time, that transfer of the β_1 Na,K-ATPase subunit gene augments Na,K-ATPase function in epithelial cells and liquid clearance in rat lungs. Conceivably, overexpression of Na,K-ATPases could be used as a strategy to augment lung liquid clearance in patients with pulmonary edema. (*J. Clin. Invest.* 1998. 102:1421–1430.) Key words: Na,K-ATPase • adenovirus • gene transfer • lung liquid clearance • lung epithelial cells

Introduction

Edema accumulates in the alveolar airspace due to changes in capillary permeability and/or imbalances between Starling's forces (1). It is now recognized that, in contrast to its formation, alveolar edema is removed by the active transport of Na^+ out from the airspaces into the interstitium and capillaries (2, 3). This active transport creates a transepithelial osmotic gradient that causes water to move out of the airspace. Lung epithelial Na,K-ATPases have been shown to contribute to active Na^+ transport and lung edema clearance (2–5).

Na,K-ATPases are transmembrane heterodimers composed of α and β subunits (6). The α subunit is a transmembrane protein that cleaves high-energy phosphate bonds and exchanges intracellular Na^+ for extracellular K^+. The smaller β subunit is a glycosylated transmembrane molecule that controls α/β heterodimer assembly and insertion into the plasma membrane; its presence is required for normal Na,K-ATPase function. In the alveolus, Na,K-ATPases are located in alveolar type 2 epithelial cells (AT2)[1] and alveolar type 1 epithelial cells (7–10). This multimeric "pump" works in concert with other epithelial transport proteins, including apical Na^+ channels, to clear alveolar edema (11–13). AT2 cells and whole rat lungs express the α_1 and β_1 subunits of this multigene family. Levels of Na,K-ATPase mRNA and protein change in response to edemagenic stimuli such as hyperoxia (5, 10). These changes parallel changes in active Na^+ transport and lung edema clearance (5, 14, 15). These findings were associated with increases in Na,K-ATPase function in AT2 cells (5). Similar findings have been reported after treatment of alveolar epithelial cells and lungs with dopamine, dobutamine, and isoproterenol (16–18). These studies show that increased Na,K-ATPase expression is associated with increased Na,K-ATPase function and lung liquid clearance, suggesting that alveolar Na,K-ATPases play an important role in keeping the alveolus free from edema.

We reasoned that overexpression of Na,K-ATPase subunit genes could be used to enhance Na^+ pump abundance in lung epithelial cells and lung edema clearance in rat lungs. To test this hypothesis, we engineered recombinant, replication-deficient human type 5 adenoviruses containing cDNAs for the rat Na,K-ATPase α_1 and β_1 subunits. We tested these viruses in isolated rat AT2 cells, airway epithelial cell monolayers, and physiologic experiments in isolated rat lungs. The results of this study show that adenoviral-mediated overexpression of the Na,K-ATPase β_1 subunit increases Na,K-ATPase function in lung epithelial cells and lung liquid clearance in rats.

Methods

Shuttle vector construction

The expression cassette of pCMVβ (Clontech, San Francisco, CA) was inserted into the XbaI site of pXCX2, a pBR322 based plasmid containing the left end (map units 0–2 and 9.24–17.24) of the human adenovirus type 5 (a gift from F. Graham, McMaster University, Hamilton, Ontario, Canada) to produce pMRCMVβ-gal (19). This expression vector contains the immediate early promoter and en-

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1. *Abbreviations used in this paper:* AT2, alveolar type 2 epithelial cells; BSA, buffered salt albumin; CMV, cytomegalovirus; CPE, cytopathologic effect; EBD, Evans blue dye; LDH, lactate dehydrogenase; pfu, plaque forming units.

hancer from CMV, a full length cDNA for *Escherichia coli lacZ* and the SV40 t intron polyadenylation signal. The β-galactosidase cDNA was excised from pMRCMVβ-gal and replaced with full length cDNAs for the rat α_1 and β_1 Na,K-ATPase subunits to produce pMRCMVα_1 and pMRCMVβ_1, respectively. A similar shuttle vector was produced that contained no cDNA (pCMVNull).

Adenovirus construction

A 40.3-kb plasmid containing a human type 5 adenovirus (dl 309) genome (pJM17)(20) without the E1a gene was cotransfected (Lipofectin; GIBCO BRL, Gaithersburg, MD) with the above described shuttle vectors into human embryonic kidney cells (ATCC 293; American Type Culture Collection, Rockville, MD). Homologous recombination, viral assembly, and replication were detected by the development of cytopathologic effect (CPE). Cells from plates showing CPE were collected and disrupted by six cycles of freezing and thawing. This crude viral lysate was expanded in 293 cells. After repeat development of CPE, PCR was used to confirm the presence of the appropriate cDNAs and cytomegalovirus (CMV) promoter in the lysate. PCR positive cultures were plaque-purified three times in 293 cells before large scale amplification (19, 21).

Adenovirus propagation and purification (19)

Subconfluent 15-cm tissue culture plates were infected with three plaque-forming units (pfu) per cell. After development of CPE, the cells were harvested, concentrated, and disrupted with six cycles of freezing and thawing. The resultant cell lysate was cleared of cellular debris by centrifugation before purification through serial CsCl density gradient centrifugations. The resultant virus was dialyzed against 10 mM Tris HCl, pH 7.4/1 mM MgCl/10% glycerol to remove CsCl before storage in 10% glycerol at −70°C. Viral titers were ascertained by the enumeration of plaques produced by adenovirus in 293 cells grown under agarose (21). Viral purity was assessed by demonstrating the absence of detectable wild-type DNA by PCR and plaque production in A549 cells grown under agarose.

Epithelial cell isolation

AT2 cell isolation. The use of animals for this study was approved by the Michael Reese Hospital Institutional Animal Use and Care Committee (protocol #930104; Chicago, IL). AT2 cells were isolated from pathogen-free male Sprague Dawley rats (200–225 g; Indianapolis, IN) as previously described (5, 7, 22). After systemic anticoagulation with heparin and induction of general anesthesia with pentobarbital, the lungs and mediastinum were removed en bloc. The lungs were perfused via the pulmonary artery, lavaged, and digested with elastase (30 U/ml; Worthington Biochemical, Freehold, NJ). The AT2 cells were purified from alveolar macrophages by differential adherence to IgG pretreated dishes. Non-adherent cells were suspended in DME medium (Irvine Scientific, Irvine, CA) containing 10% FBS (Hyclone Inc., Logan, UT) with 2 mM L-glutamine (Irvine Scientific, Irvine, CA), 40 μg/ml gentamicin, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma Chemical Co., St. Louis, MO). For studies of epithelial cell ion transport and assessment of cytotoxicity, 3.5×10^5 cells in 2 ml of serum-containing medium were plated into each well of 6-well tissue culture plates (Falcon, Franklin Lakes, NJ). For Northern and Western blot studies, 10^7 cells were plated on 10-cm tissue culture dishes (Corning Glassworks, Corning, NY). Cells were incubated in a humidified atmosphere of 5% CO_2/95% air at 37°C. Cells were allowed to adhere for 24 h before infection with adenovirus. Plating efficiency and cell viability were 50–60% and 95%, respectively, 24 h after plating.

Rat airway epithelial cell isolation. Rat tracheal cells were isolated by enzyme digestion as previously described (23). Freshly isolated cells were seeded at a density of 3×10^5 cells/cm^2 onto rat tail collagen-coated semipermeable membranes (0.6 cm^2, Millipore-Inserts; Millipore Corp., Bedford, MA). The cells were maintained at 37°C in a humidified atmosphere of 7% CO_2 and air. 24 h after plating, the mucosal media was removed and the cells were allowed to grow at the air–liquid interface (24). The culture medium used was Jokliks MEM (GIBCO BRL). On average, the epithelia were dry by 3 d after seeding, and had transepithelial resistances of $> 800\ \Omega$.

Adenovirus infection protocol

AT2 cells. Cells were washed three times with DME/2% FBS (infection medium) before application of adenovirus in 1–2 ml of infection medium. Plates were intermittently rocked for 2 h after infection, whereupon 3–7 ml of DME/10% FBS with antibiotics was added (19, 25). Preliminary experiments were conducted using moi's of 0–100 of adMRCMVβ_1. The results of these studies revealed diminished cell viability beyond 72 h when > 50 live viral particles/cell were used.

Rat lungs. Rats were anesthetized with 40 mg/kg of pentobarbital intraperitoneally and orally intubated with a 14-g plastic catheter (Becton Dickinson, Sandy, UT). Five experimental groups were studied: surfactant (n = 4), adCMVNull (n = 4), adMRCMVβ-gal (n = 4), adMRCMVα_1 (n = 6) and adMRCMVβ_1 (n = 7). A mixture of adenovirus in a 50% surfactant (Survanta, Abbott Laboratories, Columbus, OH)/50% dialysis buffer vehicle was administered in four aliquots of 200 ml (26). Rats were rotated 90° between instillations given at 5-min intervals. Immediately before instillation of vehicle-forced exhalation was achieved by circumferential compression of the thorax. Compression was relinquished after endotracheal instillation of 200 μl of virus/vehicle followed by 500 μl of air. This resulted in a forceful inspiration that facilitated adenoviral dispersion to the distal airspaces. Rats were allowed to recover before extubation. Infected animals were maintained in separate isolator cages within a biosafety level 3 facility.

Rat airway epithelial cells. 7 d after plating, the epithelia were transfected as previously described (24). 50 moi in 50 μl of PBS of either adMRCMVβ-gal or adMRCMVβ_1 were added to the apical side of the epithelia. After 12 h, the viruses were removed, the epithelia were rinsed and incubated for 48 to 72 h before assay.

Documentation of β-galactosidase expression.

AT2 cells. 24 h after infection, AT2 cells were washed with PBS, pH 7.4, and fixed for 15 min at 4°C with 2% formaldehyde/0.2% glutaraldehyde in PBS. A mixture of 5 mM $K_4Fe(CN)_6$-$3H_2O$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$ in PBS with 0.5 mg/ml of X-gal (Sigma Chemical Co.) was applied to the cells (19). The reaction was allowed to proceed overnight at 37°C before a final wash with PBS. Cells were visualized directly in the tissue culture dish with an inverted phase microscope. Transfection efficiency (% cells infected) was determined as the number of cells with blue cytoplasm/100 cells (27, 28) All experiments were performed in triplicate. Out-of-phase (11 phase ring) photomicrographs were taken of all specimens at 200×. Photomicrographs were digitized and stored on CD-ROM. The digital images were assembled and color corrected (Kodak E6 color correction algorithm) using Photoshop (Adobe Systems Incorporated, San Jose, CA).

Rat lungs infected with adenovirus. Rat lungs infected with adenovirus were stained in similar fashion after completion of the isolated lung experiments. Lungs were repeatedly lavaged with PBS to remove instilled tracers before the instillation of 0.2% glutaraldehyde/2% formaldehyde for 20 min at 4°C. Fixative was removed by lavage with PBS followed by instillation of the above described X-gal solution. X-gal staining was allowed to proceed overnight at 37°C. The lungs were again lavaged with PBS before instillation of 1% buffered formalin. Representative portions of the upper, middle, and lower portions of both lungs were then imbedded in paraffin for sectioning. Sections (5 μm) were analyzed without counterstaining and photographed at 200× as described above.

Rat airway epithelial cells. Rat airway epithelial cells were stained with X-gal as described above for AT2 cells.

Northern blot analysis

To determine the steady state levels of mRNA transcripts of rat α_1 and β_1 Na,K-ATPase in rat AT2 cells after infection with replication deficient adenovirus, Northern blot analyses were performed (19, 27, 29). 5 μg of total RNA was size fractionated through 1% agarose/

MOPS/1.7 M formaldehyde gels by electrophoresis, transferred to nylon membranes (Nytran, Schleicher & Schuell, Keene, NH) by capillary action and bound by ultraviolet cross-linking. Species and subunit specific ^{32}P-labeled cDNA probes for hybridization were generated by random priming. Nylon membranes were then hybridized at 65°C for 36 h in 0.5 M Na_2HPO_4 (pH 7.0), 1 mM EDTA, 0.5% BSA, and 7% SDS. Membranes were twice washed for 15 min at room temperature with 2×SSC/0.1% SDS followed by two washes with 0.5×SSC/0.1% SDS. Two 20-min final washes at 65°C were performed using 0.1×SSC/0.1% SDS. Membranes were then exposed to X-ray film (Biomax MR, Eastman Kodak Co., Rochester, NY) at −80°C for 16–24 h before development. To demonstrate similarity of lane loading, the membranes were stripped of Na,K-ATPase probes and rehybridized with a ^{32}P-labeled rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe and re-exposed to X-ray film. Message expression was quantified using a linear scanning laser densitometer (Molecular Dynamics Corporation, Sunnyvale, CA).

Western blot analysis

a) AT2 cells infected with moi's of 5 or 10 of adMRCMVβ-gal or adMRCMVβ_1 were washed with PBS before in situ lysis with 10 mM Tris-Hepes/3 mM EGTA/1 mM EDTA/2 mM DTT/10 mM mannitol with 0.01 mg/ml *N*-tosyl-L-phenylalanine chlorylmethyl ketone, 0.1 mM PMSF, 0.01 mg/ml leupeptin (4°C) (Sigma Chemical Co.). Cells were scraped, collected, and homogenized with a Potter Ehvehjem homogenizer. The cell homogenate was centrifuged at 1,500 *g* for 15 min, and the resultant supernatant was collected and centrifuged at 100,000 *g* for 1 h. The subsequent supernatant was rehomogenized and centrifuged at 1,500 *g*, before repeat centrifugation at 100,000 *g*. The pellet thus obtained was combined with the first pellet. All manipulations and solutions were at 4°C. The final pellet was resuspended in 100 μl of homogenization buffer and quantified using a Bradford Assay (Bio-Rad protein assay; Bio-Rad, Hercules, CA). Proteins were separated by SDS-PAGE and transferred to nitrocellulose (Hybond C$^+$, Amersham Corporation, Arlington Heights, IL). Nitrocellulose blots were blocked overnight at 4°C in blotto (5% w/v nonfat dry milk, 0.1% sodium azide, in 150 mM NaCl, 25 mM Hepes, pH 7.4) Anti-β antiserum raised in rabbits against purified β_1 protein from dog kidney (1:100 dilution; provided by Dr. Amir Askari, Medical College of Ohio, Toledo, OH) in 1% blotto was allowed to bind to membranes for 2 h at 37°C on a rocking table. After three 10-min washes with 1% Triton X-100 in 150 mM NaCl/25 mM Hepes, pH 7.4/ 2 μCi of ^{125}I-labeled goat anti–rabbit secondary antibody in 1% blotto were added. After 1 h at 25°C, blots were washed as before, dried, and exposed to X-ray film (19, 27).

b) The right upper lobe of each rat was harvested and frozen at −70°C before use. Specimens were processed with a Polytron homogenizer (Kinematica AG, Littau, Switzerland) after addition of 1 ml of homogenization buffer. The homogenate was cleared by centrifugation at 700 *g* at 4°C for 20 min, and the resultant supernatant was again centrifuged at 9,500 *g* at 4°C for an additional 20 min to partition cell membranes into the supernatant. Supernatants were then centrifuged at 60,000 *g* for 30 min at 4°C. The pellet was resuspended in 100 μl of homogenization buffer, and protein concentrations were assayed (Bradford, Bio-Rad, Hercules, CA). Samples were heated at 95°C for 20 min before size fractionation at 12% PAGE. Similarly processed rat brain was used as positive control for all blots. After electrophoreses gels were immersed in transfer buffer for 10 min (20% methanol/0.037% SDS/48 mM/Tris/39 mM glycine, all from Sigma Chemical Co.) at room temperature before semi-dry transfer (Bio-Rad semi-dry transfer system, Hercules, CA) at 20 V for 1 h to nitrocellulose. Membranes were treated as described above with the exception of the use of the enhanced chemiluminescence immunodetection system (Amersham Corp.).

Na,K-ATPase function in AT2 cells. Ouabain-sensitive $^{86}Rb^+$ uptake was used to estimate the rate of K^+ transport by Na,K-ATPase in AT2 cells (7, 19). Cells were incubated with and without 5 mM ouabain (ICN, Aurora, OH) for 5 min at 37°C in a gyratory bath at 100 rpm. This medium was removed, and otherwise identical fresh medium containing 1 μCi/ml $^{86}Rb^+$ (Amersham Corp., Arlington Heights, IL) was added. 5 min later the assay medium was removed by aspiration and the cells were washed with ice-cold 150 mM $MgCl_2$. Plates were allowed to dry and cells were solubilized in 0.2% SDS. $^{86}Rb^+$ influx was quantitated from aliquots of the SDS extract with a liquid scintillation counter. Protein was quantitated using the Lowry method. Initial influx is expressed as μM K^+/g of protein/min. Three data points were obtained for each condition during each experiment. All experiments were performed in triplicate.

Assessment of AT2 cell injury. Culture media concentrations of K^+ and lactate dehydrogenase (LDH) were used as indicators of AT2 cell cytotoxicity. AT2 cells were plated in 6-cm dishes for 24 h before infection with an moi of 5 or 10 of adMRCMVβ_1 or adMRCMVβ-gal. Cells were maintained in 7 ml of complete medium for 24 h before aspiration and remeasurement of medium volume. Specimens were centrifuged at 600 *g* to remove cells and cellular debris, and K^+ and LDH concentrations were measured in the resultant supernatant. K^+ was determined using an ABL620-100EML electrolyte analyzer (Radiometer Medical A/S, Copenhagen, Denmark). LDH concentrations were measured with a Hitachi 747 analyzer (Boehringer Mannheim, Indianapolis, IN). Cells from these plates were trypsinized and counted to assure uniformity of cell number.

Isolated lung experiments. Our fluid-filled isolated lung preparation has been previously described in detail (2, 5, 14, 15). Briefly, rats were anesthetized (Nembutal 65 mg/kg intraperitoneal) and ventilated via a tracheotomy for 10 min with 100% O_2. A median sternotomy was performed and the pulmonary artery and left atrial appendage were cannulated and perfused with a solution of 3% buffered salt albumin (BSA) solution. Fluorescein-labeled (FITC) albumin was added to the perfusate to monitor leakage of protein from the vascular space into the airways. 90 ml of perfusate was continuously recirculated, at constant pressure and flow, through the pulmonary circulation. Arterial and venous pressures were maintained at 8 and 0 cm H_2O, respectively, and pH was maintained at 7.40 by bubbling a mixture of 5% CO_2 and 95% O_2 through the BSA solution. The lungs were removed from the thorax and two sequential bronchoalveolar lavages were performed with 3 ml of BSA solution containing Evans blue dye (EBD; Sigma Chemical Co.), $^{22}Na^+$ (Dupont, NEN, Boston, MA), and ^{3}[H]mannitol (Dupont, NEN, Boston, MA). Additional solution was then added to achieve a total instillate volume of 5 ml. The lungs were then placed in a "pleural bath" containing 100 ml BSA solution maintained at 37°C. This allowed us to follow markers that had moved across the pleural membrane or were drained by the lung lymphatics. Samples were drawn from the three reservoirs: air-space instillate, "pleural bath," and perfusate at 10 and 70 min after starting the experiment protocol. The fraction of lung liquid clearance due to Na,K-ATPase function was estimated by adding ouabain (10^{-4} M) to the perfusate of some experiments ($n = 4$).

Calculations for isolated-perfused rat model (2, 5, 14–16). Concentration of EBD–albumin was used to estimate airspace volume. As virtually all EBD–albumin remains in the airspace, we calculate instillate volume (V) at a given time *t* as:

$$V_t = V_o [EBD]_o / [EBD]_t$$

Where V_0 is the initial volume instilled and $[EBD]_0$ and $[EBD]_t$ are the concentrations if EBD–albumin at times 0 and *t,* respectively. The removal of sodium from the alveolar space during a defined period of time is accompanied by isotonic water flux and is given by:

$$J_{Na,net} = J_{Na,out} - J_{Na,in}$$

where $J_{Na,net}$ is the net or active Na^+ transport, $J_{Na,out}$ is the total or unidirectional Na^+ outflux and $J_{Na,in}$ is the back flux of Na^+ into the alveolar fluid by passive bidirectional movement. Because Na^+ concentration remains constant in all compartments, the net Na^+ flux (which we refer to as active Na^+ transport) from the airspace is:

$$J_{Na,net} = (V_0 - V_t)\,[\,Na^+\,]/t$$

The unidirectional fluxes of Na^+ from the alveolar space, a result of active transport and passive movement, was calculated as:

$$J_{Na,out} = J_{Na,net}\,\{\,(\ln\ {}^{22}Na^+{}_t / {}^{22}Na^+{}_0)\,/\,(\ln V_t/V_0)\,11\,\}$$

Similarly, the (unidirectional) volume flux of ^3H-labeled mannitol (typically expressed as PA, permeability of surface area) was calculated as:

$$PA = (V_0 - V_t)/t\,\{\,(\ln\ [^3H\text{-labeled mannitol}]_t / [^3H\text{-labeled mannitol}]_0)\,/\,(\ln V_t/V_0)\,\}$$

Albumin flux from the pulmonary circulation into the alveolar space was determined from the fraction of FITC–albumin that appears in the alveolar space during the experimental protocol. For reasons of comparison, fluxes are reported as volume fluxes (vol/time) by using the appropriate solute concentrations.

Immunohistochemistry. Sections from the upper, middle, and lower portions of each fixed lung (right and left) were imbedded in paraffin for immunohistochemical analysis. 5-μm sections were deparaffinized in xylene and rehydrated though serial ethanols. Specimens were then treated with 3% H_2O_2 to reduce endogenous peroxidase activity before blocking of background immunoreactivity with non-immune goat serum. Primary antibody (1:500 dilution, rabbit anti–dog β_1 from A. Askari) was added for 1 h at room temperature. Sections were washed with PBS before addition of goat anti–rabbit secondary antibody. Immunodetection was accomplished via 3,3′-diaminobenzidine staining for immunoperoxidase activity (Vector Elite ABC kit, Vector Laboratories, Newcastle-upon-Tyne, UK). Sections were photographed at 200× without counter staining.

Statistical analysis. All values are reported as means±standard deviation. Statistical analysis was performed using Student's *t* test comparisons of viral-infected groups to sham-infected or surfactant-treated controls. ANOVA was used for comparisons among experimental groups. Statistical significance was defined as $P < 0.05$.

Results

Transfection efficiency. AT2 cells infected with moi of 1, 5, or 10 of adMRCMVβ-Gal demonstrated a transfection efficiency of ∼ 50, ∼ 70, and ∼ 90% 24 h postinfection, respectively. Sham or adMRCMVβ_1 infected cells showed no evidence of β-galactosidase activity.

Northern blot analysis. As shown in Fig. 1, Northern blot analysis of mRNA isolated from adenoviral-infected AT2 cells using a full-length cDNA probe complementary to the rat β_1 Na,K-ATPase subunit mRNA revealed the presence of a β_1 message that migrated between the 2.37- and 1.35-kb molecular weight standards. This truncated message is consistent with the vector design of adMRCMVβ_1, which uses no endogenous polyadenylation signals. A 2.7/2.35–kb doublet, consistent with endogenous β_1 was also noted (28). Adenoviral-derived β_1 mRNA levels were greater in cells infected with an moi of 10 than an moi of 5 of adMRCMVβ_1. Duplicate blots were hybridized using a cDNA probe complementary to the rat α_1 Na,K-ATPase subunit mRNA. A 3.7-kb α_1 band was noted in all lanes. Laser scanning densitometry did not reveal any differences in α_1 mRNA expression between groups. Hybridization with rat GAPDH demonstrated equivalent lane loading of total RNA between all experimental samples.

Transgene expression. Western blot analysis of membrane fractions from AT2 cells infected with adMRCMVβ_1 demonstrated β_1 subunit immunoreactivity at ∼ 45–50 kD (Fig. 2). Relative expression was greater in the moi of 10 than moi of 5 AT2 cells. No β_1 protein expression was noted in the adMRCMVβ-Gal or sham-infected cells using these methods.

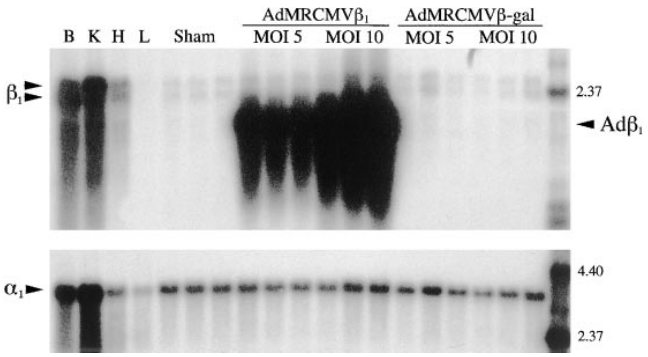

Figure 1. Northern blot analysis of triplicate samples of 5 μg of total RNA harvested from AT2 cells infected with adMRCMVβ_1. Hybridization using a full-length cDNA rat β_1 probe shows dose-dependent expression of an mRNA that migrates between the 2.35- and 1.35-kb molecular weight markers (*A*). Sham and adMRCMVβ-Gal–infected cells do not express this message. A 2.7/2.35–kb doublet, consistent with endogenous β_1, is evident in all samples. The first four lanes represent signals from 5 μg of total RNA isolated from uninfected control rat tissues (*B* = brain, *K* = kidney, *H* = heart, and *L* = liver); the right most lane is a molecular weight ladder. An otherwise identical Northern blot hybridized with a cDNA probe complementary to the rat α_1 subunit (*B*) shows no change in expression of this subunit among the experimental groups. Hybridization with rat GAPDH demonstrated equivalent lane loading of total RNA between all experimental samples.

Na,K-ATPase function (ouabain-sensitive $^{86}Rb^+$ uptake). Preliminary experiments to ascertain dose-response relationships were performed using moi's of 1–200 of adMRCMVβ_1. Maximal ouabain sensitive $^{86}R\beta_1$ uptake was noted with moi of < 10. Cells infected with moi's of 5 or 10 of adMRCMVβ_1 showed up to a 2.5±0.3–fold increase in ouabain-sensitive $^{86}Rb^+$ uptake (Fig. 3). Activity in the moi of 5 cells was not different from the moi of 10 cells. Cells infected with an moi of 1 did not demonstrate any change in ouabain-sensitive $^{86}Rb^+$ uptake. Parallel experiments using adMRCMVα_1 produced no increases in ouabain-sensitive $^{86}Rb^+$ uptake. AT2 cells infected with moi's of 1, 5, or 10 of adMRCMVβ-Gal demonstrated $^{86}Rb^+$ uptake that was not different from sham-infected controls (Fig. 3).

Transepithelial electrical properties in airway epithelial cells. Transepithelial Na^+ transport was studied in airway epithelial cells under short circuit conditions. As shown in Fig. 4 (*upper graph*), treatment with adMRCMVβ_1 resulted in greater than a twofold increase in transepithelial Na^+ transport (Isc_{Amil}) compared to saline-treated controls (2.04±0.16 μA/cm^2 versus 0.89±0.16 μA/cm^2, $P < 0.01$). adMRCMVβ-gal had no significant effect on Isc_{Amil}. To evaluate for basolateral Na,K-ATPase function, the apical membrane was permeabilized with Nystatin (360 μg/ml). This resulted in a net cation Isc from the apical to the basolateral side that was not blocked by amiloride (10^{-4} M). AdMRCMVβ_1 infection produced a greater than threefold increase in basolateral ouabain-sensitive Na^+ transport ($Isc_{Ouabain}$) compared to uninfected controls (−1.48±0.24 μA/cm^2 versus −0.48±0.12 μA/cm^2 $P < 0.01$) (Fig. 4, *lower graph*). AdMRCMVβ-gal did not affect $Isc_{Ouabain}$. Transfection efficiency in the AdMRCMVβ-gal–infected cells was ∼ 35%.

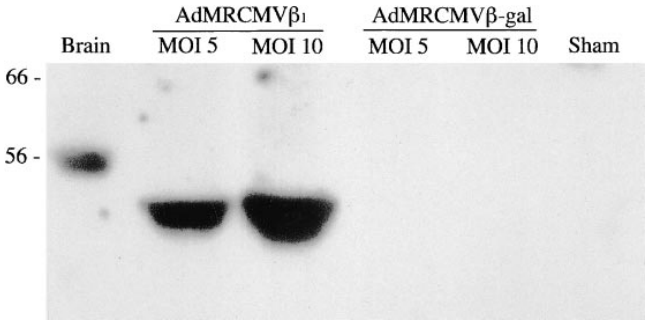

Figure 2. Western blot analysis of 30 μg of AT2 cell membrane fractions reveals dose-dependent immunoreactivity in adMRCMVβ_1–infected cells using a polyclonal anti–dog β_1 antibody. The observed band migrated beyond brain β_1 (55 kD). Molecular weights are in kD.

Assessment of cell injury. K^+ concentrations in the adMRCMVβ_1–infected cells were 5.4±0.2 and 5.1±0.3 meq/ml in the moi of 5 and 10 groups. K^+ concentrations for the sham, and adMRCMVβ-gal (moi 5 and 10) groups were: 5.3±0.2, 5.4±0.2, and 5.1±0.3 meq/ml, respectively. LDH concentrations in the adMRCMVβ_1–infected cells were 41 = ±10 and 37±4 IU in the moi of 5 and 10 groups. These values were not different than those noted in the sham (38±9 IU) and adMRCMVβ-gal (moi 5; 41±10, moi 10; 37±4) groups. To test for time-dependent effects of adenoviral-mediated Na,K-ATPase gene transfer, AT2 cells were infected with moi's of 5 or 10 of adMRCMVβ_1 and observed for 120 h. No change in cell number or gross cytologic appearance was appreciated.

Measurement of lung liquid clearance. Lung liquid clearance and epithelial permeability to Na^+, mannitol and albumin were measured 7 d after infection, using a fluid-filled, isolated lung preparation. Dose-response relationships were established by infecting rats with 10^9-10^{10} pfu of AdMRCMVβ_1. Maximal edema clearance was noted with 4×10^9 pfu of adMRCMVβ_1. As compared to the other experimental groups, alveolar liquid clearance was increased by 100% in rats given adMRCMVβ_1 (Fig. 5). No change in lung liquid clearance was noted in animals given any dose of adMRCMVα_1. As shown in Fig. 6, ouabain added to the perfusate of four rat lungs infected with AdMRCMVβ_1 decreased lung liquid clearance by ~ 75%, to a level similar to that seen in uninfected controls demonstrating that the observed increase in clearance is due to increased Na,K-ATPase function. As shown in Fig. 7,

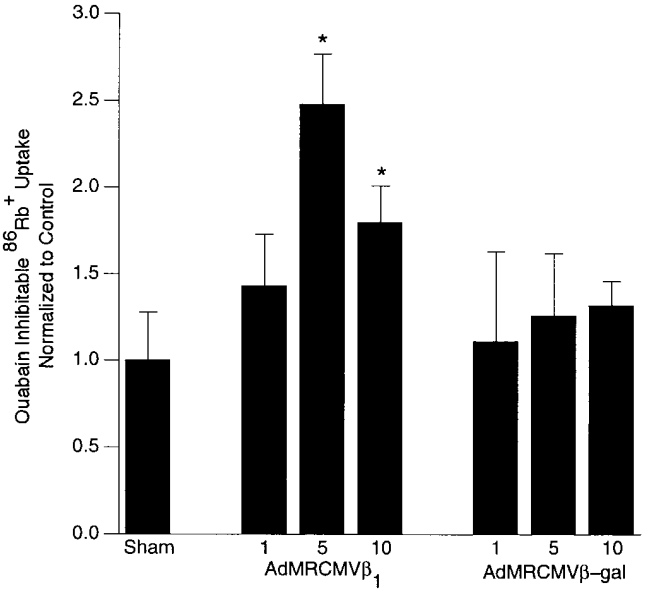

Figure 3. Na,K-ATPase function in AT2 cells was measured as ouabain-inhibitable $^{86}Rb^+$ uptake. As compared to sham-infected controls, $^{86}Rb^+$ uptake was increased 2.5±0.3– and 1.8±0.2–fold in cells infected with moi of 5 and 10 of adMRCMVβ_1, respectively. No increase in activity was noted in cells infected with adMRCMVβ-Gal or adMRCMVα_1 (data not shown). *$P < 0.001$.

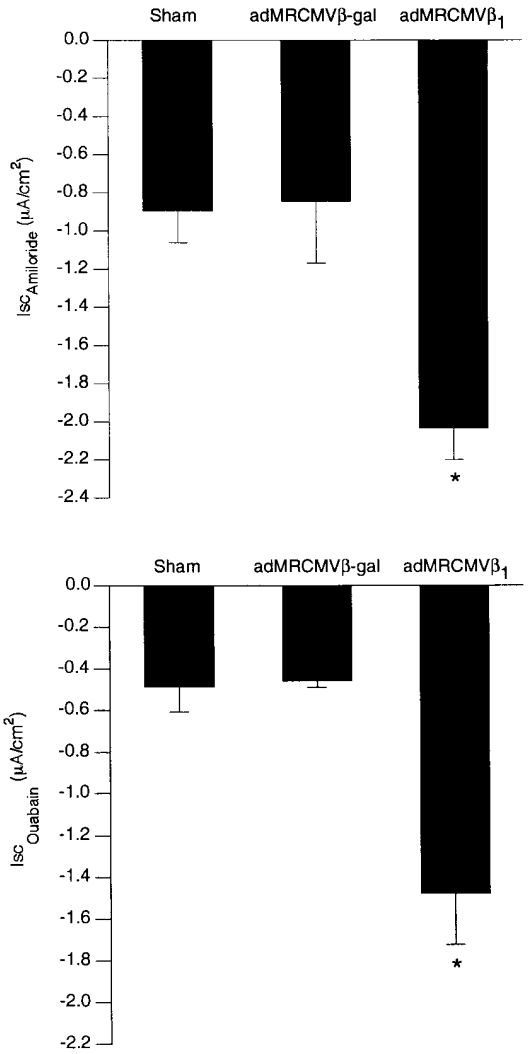

Figure 4. Amiloride-sensitive Isc in rat airway epithelial monolayers after Na,K-ATPase subunit gene transfer (*top*): As compared to sham-infected controls, Isc_{Amil} was increased 2.3-fold in cells infected with adMRCMVβ_1. No increase in Isc_{Amil} was noted in cells infected with adMRCMVβ-gal (*$P < 0.01$). As compared to sham-infected controls, ouabain-sensitive Isc in nystatin-permeabilized rat airway epithelial monolayers ($Isc_{Ouabain}$) was increased threefold in cells infected with adMRCMVβ_1 (*bottom*). Isc was measured using 9–12 filters in three separate experiments used for each condition. No increase in $Isc_{Ouabain}$ was noted in cells infected with adMRCMVβ-gal (*$P < 0.01$).

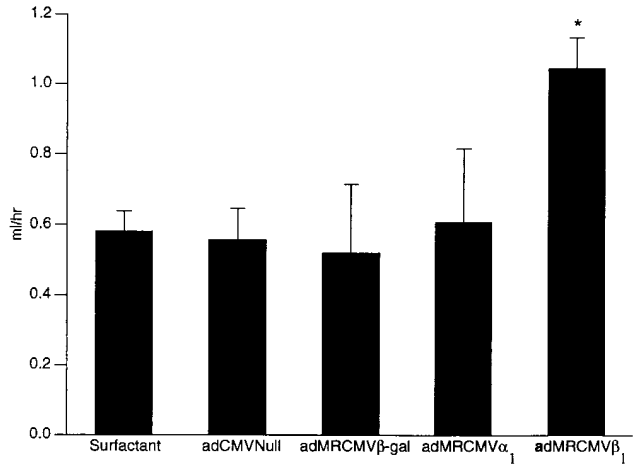

Figure 5. Lung liquid clearance is increased by ~ 100% 7 d after infection with adMRCMVβ_1. Liquid clearance in the other experimental groups was not different from surfactant-treated controls. *$P <$ 0.001.

AdMRCMVβ_1 and AdMRCMVβ-gal minimally increased $^{22}Na^+$ and ^{3}H-labeled mannitol permeability. None of the other conditions changed the passive flux of small solutes across the alveolus. The movement of FITC–albumin from the pulmonary vascular compartment into the airspaces was slightly increased in the AdMRCMVβ-gal–infected lungs only (Fig. 7). The magnitude of movement of protein tracers across the alveolar epithelial barrier was similar or less than rates reported in previous studies (2, 5, 14, 15, 18). EBD-bound albumin instilled in the airspace was not detected in the perfusate or pleural bath compartments in any of the experimental groups. This minimal movement of albumin allows us to accurately assess lung liquid clearance in this model.

Gene transfer efficiency and distribution. X-gal staining was used to demonstrate virus distribution and extent of alveolar

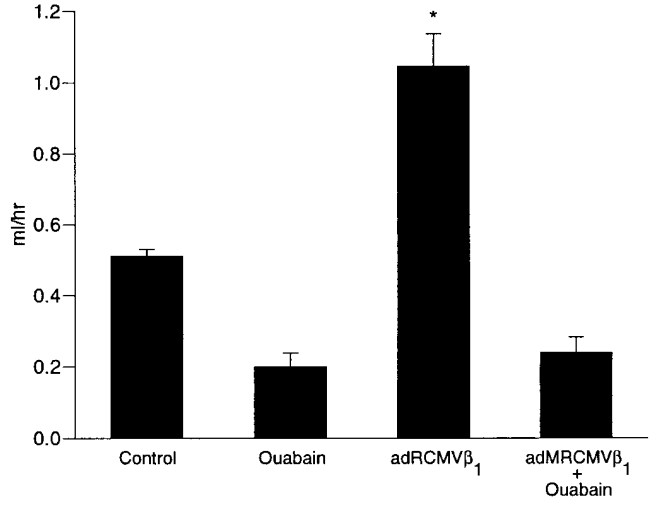

Figure 6. Lung liquid clearance was measured in adMRCMVβ_1–infected animals treated with ouabain. Ouabain (10^{-5} M) decreased lung liquid clearance by ~ 75%, to a level similar to uninfected rat lungs.

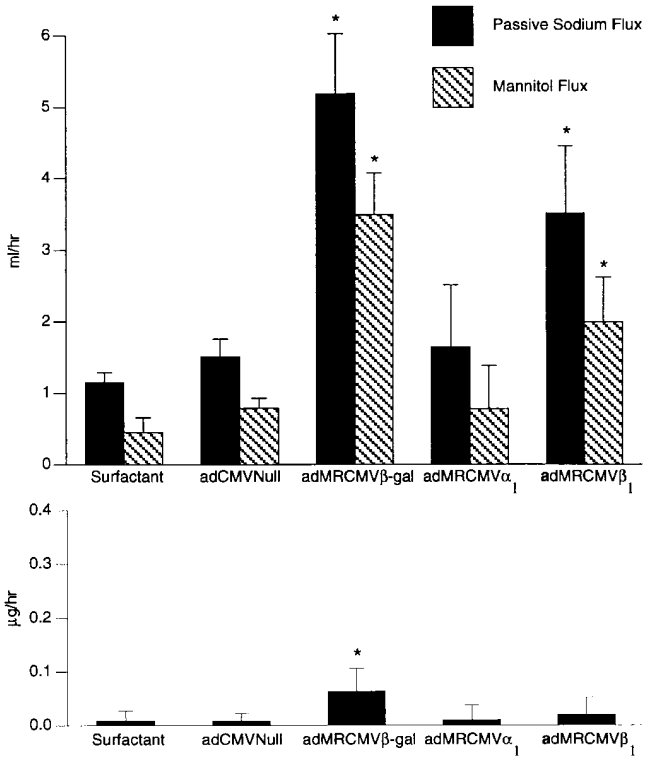

Figure 7. Epithelial permeability (*top graph*) for Na^+ (*solid bars*) and mannitol (*heavy hash marks*) was increased in rats given 4×10^9 pfu of adMRCMVβ_1 and adMRCMVβ-gal. Permeability to albumin (*lower graph*) was mildly increased in the adMRCMVβ-gal group. *$P <$ 0.01.

delivery. 4×10^9 pfu of adMRCMVβ-gal–produced *lacZ* expression in all regions of the lung (Fig. 8). No significant β-galactosidase activity was noted in lungs infected with any of the other adenoviruses. Histologic study of 5-μm sections showed expression in all sections studied (Fig. 8). Airway epithelial cells plated on semipermeable membranes and infected with adMRCMVβ-gal demonstrated β-galactosidase activity in 37.2% ±3.4 of the cells.

Transgene expression. Western blot analysis was used to confirm β_1 transgene overexpression after lung infection. Only rats given adMRCMVβ_1 (4×10^9 pfu) showed β_1 protein expression (Fig. 9). Immunohistochemical studies using the same anti–β_1 antibody showed pronounced increases in β_1 protein in the alveolar epithelium in the adMRCMVβ_1–infected animals (Fig. 9).

Discussion

Na,K-ATPases are transmembrane proteins that work in concert with other membrane-bound transport proteins to clear edema from the alveolar surface (5, 10, 11, 14, 30, 31). It has been reported that, in animal models of lung injury, changes in lung liquid clearance are associated with changes in Na,K-ATPase expression and function (5, 10, 14). Inhibition of Na,K-ATPase with ouabain has been shown to impair ion transport in cultured AT2 cells and edema clearance in isolated lungs (3, 15). Thus, this protein is an important contributor to the processes that keep the alveoli free of edema. We

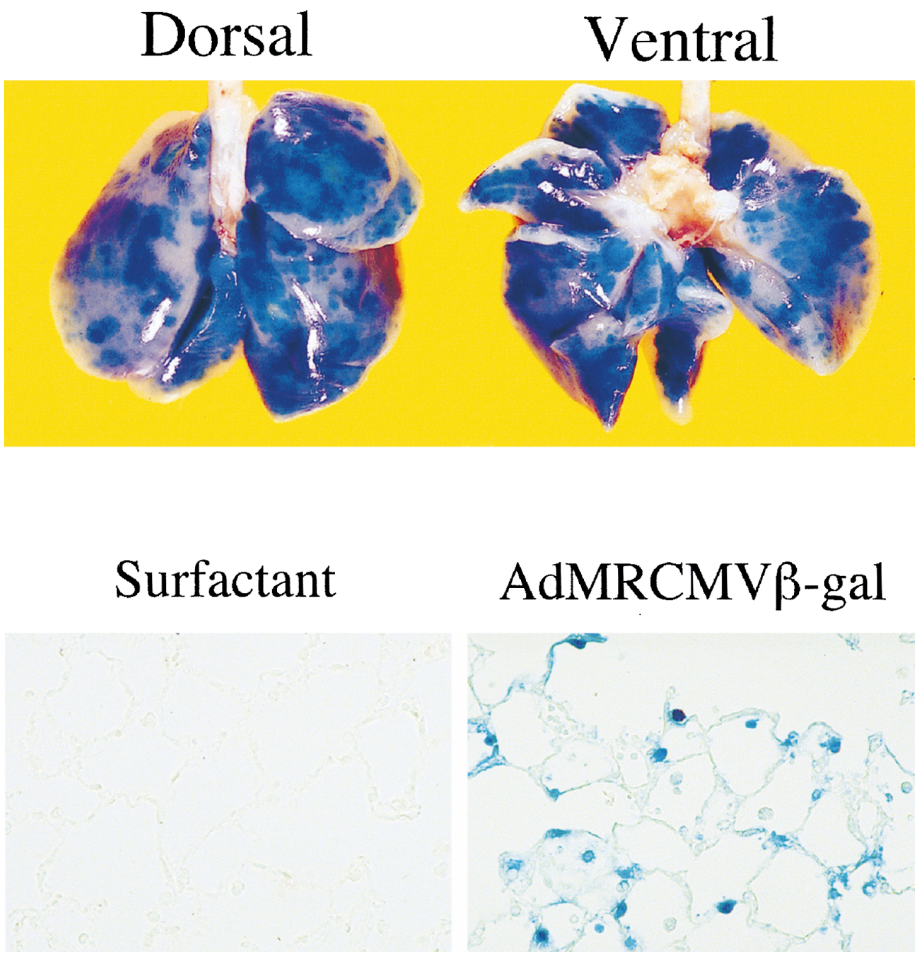

Figure 8. X-gal staining of adMRCMVβ-gal–infected lungs was used to demonstrate efficiency and uniformity of adenoviral gene transfer. Rats were given 4×10^9 pfu of adMRCMVβ-gal via tracheal instillation and were allowed to recover for 7 d before staining with X-gal solution. Gene expression is seen in all lobes. 5 μm sections of similarly processed lungs, likewise, shows widespread *E. coli lacZ* expression in most alveoli. No evidence of β-galactosidase activity was noted in rat lungs instilled with surfactant (data not shown).

hypothesized that overexpression of Na,K-ATPase subunits by gene transfer could increase Na,K-ATPase function and lung edema clearance.

The results of our in vitro experiments show that highly efficient gene transfer into AT2 cells can be achieved using low titers of replication-deficient recombinant adenoviruses per AT2 cell. These cells tolerate infection well without apparent cytotoxicity, as suggested by the normal concentrations of K^+ and LDH in the cell culture media. The observed increase in β_1 mRNA and protein demonstrate transgene activation and expression. The increased Na,K-ATPase function noted after adMRCMVβ_1 infection demonstrates that Na,K-ATPase activity in AT2 cells can be augmented via gene transfer. There was no physiologic response after α_1 subunit gene transfer. This was not due to vector design or dysfunction, as we have previously shown that our α_1-expressing virus increases Na,K-ATPase activity and abundance in a human alveolar epithelial cell line (A549) (19, 25). Thus, the absence of physiologic response after α_1 subunit gene transfer is not due to vector design or dysfunction.

To test if Na,K-ATPase subunit overexpression could increase vectorial Na^+ movement, we used a cell culture system that has been previously shown to form high-resistance monolayers (23, 24). We used these cells to study ouabain and amiloride inhibitable Na^+ transport in Ussing chambers. The results obtained in nonpermeabilized airway epithelia suggest that overexpression of the Na,K-ATPase β_1 subunit increases transepithelial Na^+ transport (Fig. 4). Permeabilization of the apical membranes allowed us to demonstrate that basolateral Na,K-ATPase function is increased in these conditions. These findings are similar to our isolated AT2 cells experiments; overexpression of the β_1 subunit increased Na,K-ATPase function.

To test whether overexpression of Na,K-ATPases in the alveolar epithelium can influence vectorial Na^+ transport and lung liquid clearance, we extended our studies to rat lungs. Using tracheal instillation of a surfactant-based vehicle, we were able to achieve homogeneous delivery of our adenoviral vector (Fig. 8) (26). We used an isolated lung preparation to measure active Na^+ transport and lung liquid clearance across the alveolar epithelium 7 d after adenoviral infection. Our data show that overexpression of the β_1, but not the α_1, subunit gene markedly increases the ability of the lung to clear edema (Fig. 5). This increase in clearance was inhibited by ouabain, suggesting that adenoviral-mediated augmentation of lung liquid clearance is due to Na,K-ATPase overexpression (Fig. 6). These findings are consistent with our cell culture data that show that the β_1 subunit overexpression increases ouabain inhibitable $^{86}Rb^+$ uptake (Na,K-ATPase function) in AT2 cells. The observed increases in β_1 mRNA and protein after AdMRCMVβ_1 infection (Figs. 2 and 9) and the absence of functional change after control virus infection suggest that the observed increases are specific responses to adenoviral-mediated gene transfer.

Glycosylation of the β subunit is a determinant of subunit lifespan and α/β heterodimer stability, and may contribute to

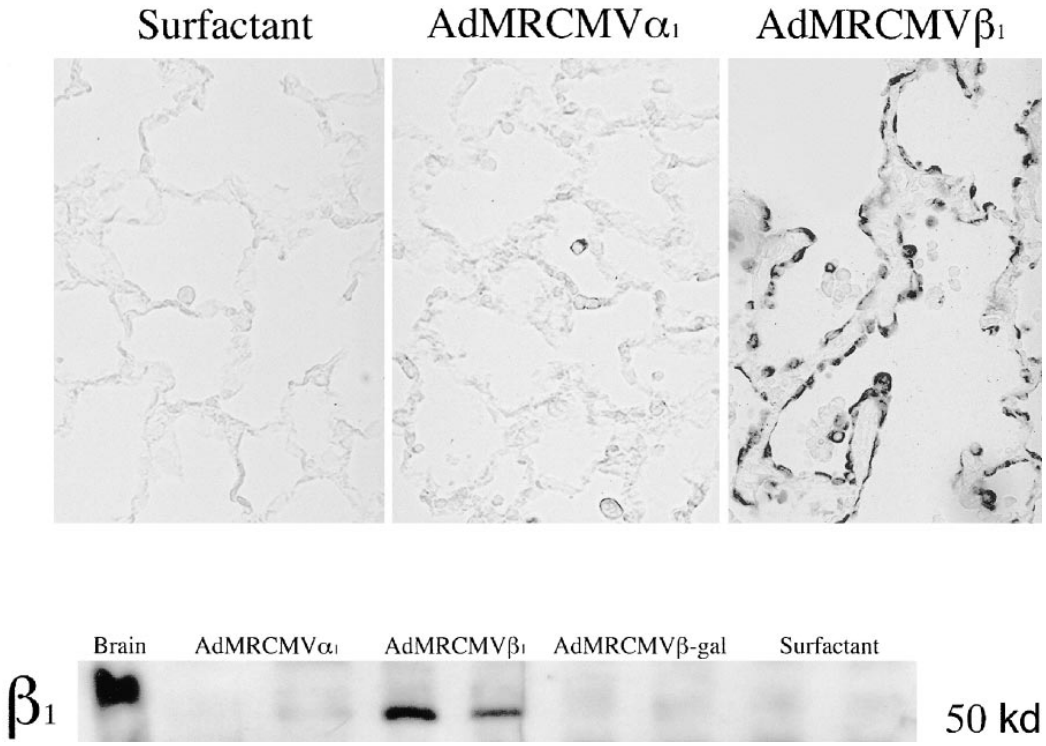

Figure 9. Western blot of whole lung tissue after Na,K-ATPase gene transfer. 50 mg of protein obtained from the right upper lobes of rats given adenovirus showed β_1 protein only in rats given adMRCMVβ_1 (4×10^9 pfu).

intracellular trafficking to the basolateral membrane of epithelial cells (32). The reported size of the protein core of the β_1 subunit is 35 kD (32, 33). Using a polyclonal anti–dog β_1 subunit antibody, we noted immunoreactivity that corresponds to a protein of $\sim$ 45–50 kD (Fig. 2). Similar findings have been reported using Sf-9 cells infected with a baculovirus that expresses a rat β_1 cDNA (34). The observed size of our adenovirus β_1 protein suggests that our transgene product is glycosylated, albeit differently, than rat brain β_1 protein. The lack of observable endogenous β_1 protein by immunoblotting techniques has been noted previously in AT2 cells (32). The observed increase in β_1 protein levels were associated with significant increases in ouabain-inhibitable $^{86}Rb^+$ uptake after infection with moi's of 5 or 10 of adMRCMVβ_1 (Fig. 3). Rat lungs infected with adMRCMVβ_1 had increased amounts of β_1 protein assessed by Western blotting and immunohistochemical techniques (Fig. 9).

The β subunit is thought to control Na,K-ATPase heterodimer assembly in the endoplasmic reticulum (ER), signaling for ER exiting and direct intracellular trafficking of assembled α/β heterodimers to the basolateral membrane in epithelial cells (35, 36). β subunits may also control the K^+ kinetics that, in part, determine net active cation movement (37). In some cell lines and under specific experimental conditions, the β subunit is rate limiting for Na,K-ATPase function. McDonough et al. (38) have shown that pig renal cells (LLC-Pk_1) maintained in hypokalemic conditions increased Na^+ pump activity. This increased activity was associated with transcription of β_1 mRNA and accumulation of newly synthesized α and β subunits. These investigators concluded that, under hypokalemic conditions, the β_1 subunit was rate limiting in these cells (39). Shanbaky and Pressley have recently shown that rat α_1 subunit can be stably expressed in monkey kidney cells (COS-1) (40). These cells did not show a net increase in Na,K-ATPase activity, but did demonstrate competition between the endogenous monkey α_1 and transgenic rat α_1 protein for available β_1 subunits, suggesting that the β_1 subunit may be rate limiting. In the studies presented herein, we observed no functional change after α_1 gene transfer. Our prior experience with adMRCMVα_1 suggests that dysfunctional transgene activation or processing of adMRCMVα_1 are unlikely explanations for this finding (19, 25). Thus, our data support the hypothesis that the β_1 subunit may be the rate-limiting Na,K-ATPase subunit in the rat alveolar epithelium.

Gene transfer is a unique tool to test what role overexpression of Na,K-ATPase genes has on Na,K-ATPase activity in lung epithelial cells and liquid clearance in lungs. Augmentation of Na,K-ATPase function by hormones (aldosterone, thyroid hormone, insulin, insulin-like growth factor, and glucocorticoids) and catecholamines (terbutaline, isoproterenol, dopamine, and dobutamine) have been reported (1, 18, 41–43). These agents have been reported to increase Na,K-ATPase function via a variety of mechanisms. Gene transfer has been used to study Na,K-ATPase expression and subunit interregulation in mammalian cell lines (34, 44–49). Increased mRNA expression and altered protein profiles have been noted in these studies. However, unlike this study, no increase in transport activity was reported. No prior study has tested what impact Na,K-ATPase gene transfer has on lung edema clearance.

Replication-deficient, recombinant adenoviruses can be used to transfer genes to eukaryotic cells and organs (50). These vectors are tropic for respiratory epithelium, can be grown in large, pure quantities; do not replicate or insert into the host genome; and are capable of efficient gene transfer (50–53). Incorporation of viral promoters can yield high level, transient expression of transgene mRNA and protein in eukaryotic cells (49). Concerns have arisen regarding possible cytotoxic and inflammatory effects of recombinant adenovirus infection. Lung inflammation has been noted in rats, non-

human primates, and humans (54). It has been suggested that E3 deletion mutants are associated with increased inflammation in cotton rats (54, 55) and that whole lung inflammation is viral-dose dependent and may be due to toxicity of viral capsid proteins (53–56). A single study has reported increased IL-8 production in human bronchoalveolar carcinoma cells (A549) infected with a nuclear-targeted β-galactosidase–expressing adenovirus (51). Pilewski et al. reported no changes in ICAM-1 expression in human bronchial epithelial cells after adenovirus infection (51, 52). Our observations of normal cytologic appearance, K^+, and LDH concentrations in culture media support the notion that these viruses, at low moi, do not produce direct cytotoxic responses in vitro. Thus, we believe that the increased Na,K-ATPase function noted in AT2 cells is due to transgene expression and is not a non-specific response to adenoviral infection.

Unlike our cell culture experiments, we noted significant host responses after delivery of adenovirus to rat lungs. Preliminary studies of rats studied 3 d after adenoviral infection showed generalized increases in alveolar epithelial permeability (data not shown). These observations caused us to allow the animals to recover for 7 d to allow alveolar permeability to improve. As can be seen from the Western blot and immunohistochemistry data, transgene levels remained high at this time point (Fig. 9). Lung liquid clearance in the animals allowed to recover for 7 d was increased by 100% in the adMRCMVβ_1 rats. Epithelial permeability to Na^+ and mannitol was minimally increased in rat lungs infected with adMRCMVβ-gal and adMRCMVβ_1, and albumin permeability was minimally increased only in the adMRCMVβ-gal rats. The absence of significant increases in alveolar albumin concentrations suggest that the changes in permeability are mild and do not confound our ability to accurately assess lung liquid clearance (15). In previous studies it has been shown that isoproterenol increased lung liquid clearance and active Na^+ transport (18, 43). These changes were associated with mild increases in paracellular permeability for small solutes (18). The absence of changes in lung liquid clearance after administration of non-β_1–expressing viruses in this study support the conclusion that overexpression of the Na,K-ATPase β_1 subunit increases lung liquid clearance.

The findings of increased Na,K-ATPase function in AT2 cells, airway epithelial cells, and rat lungs infected with adMRCMVβ_1 are encouraging. The vectors used in this study appear to be useful for studying the effects of increased Na,K-ATPase function in vitro and in vivo and may prove useful in other cell types and organs. The results of this study show that gene transfer of a single Na,K-ATPase subunit gene can increase Na,K-ATPase function in epithelial cells and rat lungs and that this increased function is associated with the physiologic effect of increased ability of the lung to clear edema. These data also support the concept that β_1 may be the rate-limiting subunit in the alveolar epithelium of rats. This data should be of importance for the design of therapeutic strategies that use gene transfer of Na,K-ATPase subunits to lungs of higher order mammals to accelerate lung edema clearance.

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