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Pulmonary oedema fluid induces non-α-ENaC-dependent Na^+ transport and fluid absorption in the distal lung

Bijan Rafii*, Daniel J. Gillie†, Chris Sulowski*, Vicky Hannam*, Tony Cheung*, Gail Otulakowski*‡, Pierre M. Barker† and Hugh O'Brodovich*‡§

*Lung Biology Programme of the Research Institute of The Hospital for Sick Children, ‡Departments of Paediatrics and §Physiology of the University of Toronto, Toronto, Ontario, Canada M5G 1X8 and †Department of Pediatrics at the University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7220, USA

To determine if pulmonary oedema fluid (EF) alters ion and fluid transport of distal lung epithelium (DLE), EF was collected from rats in acute heart failure. EF, but not plasma, increased amiloride-insensitive short circuit current (I_{sc}) and Na^+–K^+ ATPase protein content and pump activity of DLE grown in primary culture. Inhibitors of Cl^- transport or cGMP-gated cation channels had a significant ($P < 0.05$), but limited ability to block the increased I_{sc}. EF increased amiloride-insensitive, but not amiloride-sensitive, DLE apical membrane Na^+ conductance. The level of mRNA encoding epithelial sodium channel (ENaC) subunits was unchanged (α, β), or decreased (γ, $P < 0.05$) in EF-exposed DLE. EF also induced an amiloride-insensitive increase in the potential difference across murine tracheal cysts. Distal lung explants from late gestation wild-type and α-ENaC-deficient fetal mice, which normally expand due to liquid secretion, decreased in size due to liquid absorption when exposed to EF. Trypsin digestion or heat treatment of EF abrogated the ability of EF to increase amiloride-insensitive I_{sc} in DLE and liquid absorption by distal lung explants. Thus proteins or protein-dependent factors within cardiogenic EF induce an α-ENaC-independent and amiloride-insensitive apical membrane Na^+ conductance and liquid absorption in the distal lung.

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Corresponding author H. O'Brodovich: Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada, M5G 1X8. Email: hugh.obrodovich@sickkids.ca

Pulmonary oedema results from elevated transvascular pressure gradients, as occur in heart failure, or from increased vascular permeability to solutes, as occurs in the premature and adult respiratory distress syndromes. It is important to study mechanisms involved in pulmonary oedema clearance, as little is gained if one removes the cause of the oedema and the lungs cannot clear the alveolar fluid. Indeed, adults with cardiogenic or non-cardiogenic oedema survive if they have active absorption of airspace fluid, yet die if they show no evidence of active fluid clearance (Matthay & Wiener-Kronish, 1990). The Na^+ and fluid transport pathways required to clear alveolar pulmonary oedema are incompletely understood and the specific effects of pulmonary oedema fluid itself on the ion and fluid transporting ability of the distal lung are unknown.

The predominant mechanism for alveolar fluid clearance is epithelial active transport of Na^+ with linked movement of Cl^- and water. Na^+ crosses the apical membrane and is then extruded across the basolateral membrane by Na^+–K^+ ATPase. Although it is known that Na^+-permeable ion channels are the apical membrane pathway for Na^+, there is controversy regarding the nature of the Na^+ channel activity within the lung (for review see Matalon & O'Brodovich (1999) and the Discussion). The following study demonstrates that cardiogenic pulmonary oedema fluid (EF) itself alters the ion transport phenotype of distal lung epithelium (DLE) and induces the expression of an α-ENaC-independent Na^+ apical membrane conductance and fluid transport in the distal regions of the perinatal lung.

METHODS

Isolation and culture of DLE

Distal lung epithelia were isolated and cultured as described previously (O'Brodovich *et al.* 1990; Compeau *et al.* 1994) from 20-day-gestation Wistar rats (Charles River, St Constant, Quebec, Canada; breeding day = day 0, term = 22 days). DLE were then seeded at 1×10^6 cells cm^{-2} onto 0.4 μm pore size Snapwell cell culture inserts (Corning Costar, Cambridge, MA, USA) for Ussing chamber studies, and 0.5×10^6 cells cm^{-2} onto 75 mm diameter, 0.4 μm pore size Transwell cell culture inserts (Corning Costar) for subsequent protein and RNA isolation. All cells were initially submersion cultured in Dulbecco's modified Eagle's medium (DMEM; 4.5 g l^{-1} glucose with 2 mM L-glutamine and 110 mg l^{-1} sodium pyruvate) supplemented with 10% fetal bovine serum (FBS, Cansera, Rexdale, Ontario, Canada), 100 U ml^{-1} penicillin G sodium and 100 μg ml^{-1} streptomycin sulfate. All cell culture reagents were purchased from Gibco BRL (Burlington, Ontario, Canada). The culture medium was replaced

24 h after seeding to remove unattached cells, and then 24 h later, at which point the medium was replaced by either fresh DMEM with FBS (referred to as control medium), EF, bronchoalveolar lavage fluid, serum or plasma. All groups, including the control medium, were manipulated in exactly the same manner. Unless otherwise indicated, 24 h later the DLE monolayers were either mounted on an Ussing chamber or had protein or RNA extracted for Western or Northern analyses. In order to conduct dose–response experiments some DLE monolayers were exposed to various dilutions of EF. The pH at the end of the 24 h EF culture period was measured and was found to be 7.3 ± 0.1 (n = 2) and osmolarity was 345 ± 0.5 and 316 ± 5.4 mosmol l^{-1} in the medium and EF, respectively (n = 3).

Animal protocol approval

All experiments involving animals were reviewed and approved by either the Hospital for Sick Children's Animal Care Committee or the University of North Carolina's Institutional Animal Care Users Committee.

EF, plasma, serum and lavage fluid preparation

Male Sprague-Dawley rats (400 g) were anaesthetized using ketamine (100 mg ml^{-1}) and xylazine (20 mg ml^{-1}). A tracheostomy was performed and assisted ventilation (F_{I,O_2} = 1.0) was commenced using a Harvard rodent ventilator. A thoracotomy was performed and the aorta was clamped for 30 s during which time 15 ml kg^{-1} Ringer solution was infused. The resultant EF was collected through a catheter within a few minutes and the rat was killed with an overdose of anaesthetic. Serum and plasma were obtained by centrifuging clotted or heparinized blood at 10 000 g for 30 min at 4 °C. The EF was centrifuged at 10 000 g for 30 min at 4 °C. Plasma, serum and the EF supernatant were stored in aliquots at −85 °C. Prior to each experiment, EF was thawed and filtered through a 0.45 μm Millex-HA (Millipore, Nepean, Ontario, Canada) and penicillin G sodium and streptomycin sulfate were added to a final concentration of 100 U ml^{-1} and 100 μg ml^{-1}, respectively. Bronchoalveolar lavage (BAL) fluid was obtained by instilling 10 ml DMEM into the trachea of normal rats with subsequent recovery using a syringe attached to a catheter, and samples were processed as described for the EF.

To determine the heat sensitivity of the active factor(s) within the rat pulmonary EF, the fluid was heated at 80 °C for 20 min and centrifuged at 10 000 g for 10 min at 4 °C before use. In separate experiments EF was digested with 1 mg (10 000 BAEF units) of trypsin (Sigma Aldrich, Mississauga, Ontario, Canada) per milligram of total protein at 37 °C for 1 h. Subsequently, the digestion was stopped by adding appropriate amounts of soybean trypsin inhibitor (SBTI) as recommended by the manufacturer. As a control for these experiments, some DLE cells were treated with EF containing 1 mg bovine serum albumin per milligram of total protein and an equivalent amount of SBTI.

Ussing chamber experiments

The bioelectric properties of the DLE monolayers were determined as described previously (O'Brodovich *et al.* 1990; Compeau *et al.* 1994) using modified Ussing chambers (World Precision Instruments, Sarasota, FL, USA), while the cells were bathed in 37 °C Hanks' balanced salt solution (Gibco BRL) supplemented with 1.8 g l^{-1} sodium bicarbonate and equilibrated with a 5 % CO_2–95 % O_2 gas mixture. Unless stated otherwise, DLE monolayers were maintained under open circuit conditions and their short circuit current (I_{sc}) was determined using a voltage–current clamp (Physiologic Instruments, San Diego, CA, USA). The transepithelial resistance (R) was calculated by dividing the transepithelial potential difference (PD) by the I_{sc}. Values stabilized approximately 10 min after insertion of monolayers into the Ussing chambers.

The amiloride-sensitive and -insensitive I_{sc} were determined by exposing the apical side of the monolayers to a final concentration of 10^{-4} M amiloride (Sigma Aldrich). Cyclic nucleotide-gated cation channel (CNGC) activity was ascertained by exposing the DLE apical membrane to 300 μM L-*cis*-diltiazem (a gift from Dr Sandra Guggino, Johns Hopkins University, Baltimore, MD, USA) after the addition of amiloride to the bathing solution. Potassium channel activity was ascertained by the addition of 3 mM $BaCl_2$ to the basolateral side of the DLE. Additionally, Cl^- secretion was assessed using various known Cl^- transport inhibitors: basolateral 0.1 mM bumetanide (Sigma Aldrich), apical 1 mM diphenylamine-2-carboxylate (DPAC, Fluka Chemicals, Ron Kon Koma, New York, USA), apical 1 mM niflumic acid (Sigma Aldrich), apical 0.5 mM 5-*nitro*-2-(3-phenylpropylamino) benzoic acid (NPPB), apical 0.5 mM glibenclamide (Sigma Adlrich) and apical 1 mM cadmium (Sigma Aldrich). Phloridzin (1 mM, Sigma Aldrich) was added apically to assess Na^+–glucose symport activity and apical flufenamic acid (50 μM, Sigma Aldrich) was used in some experiments in view of its reported ability to block non-selective cation and Ca^{2+}-activated Cl^- channels. The role of HCO_3^- in EF-induced ion transport was ascertained by conducting experiments where DLE monolayers were bathed in HCO_3^--free, Hepes (25 mM) buffered solution oxygenated with 100 % O_2.

The ability of antioxidants to abrogate the pulmonary oedema's effect was studied through the use of 30 μM EUK-8 (Eukarion, Bedford, MA, USA) and 20 mM *N*-acetyl-cysteine (Bristol Laboratories of Canada, Montreal, Quebec, Canada). At these concentrations these two reagents were previously shown to be effective antioxidants (Compeau *et al.* 1994; Rafii *et al.* 1998).

Measurement of Na^+–K^+ ATPase

To determine the maximum Na^+–K^+ ATPase activity, 50 μM of nystatin was added to the apical side of the DLE monolayers and after the transepithelial PD reached a plateau value, the basolateral side of the monolayers was exposed to 1 mM ouabain.

Apical membrane Na^+ conductance

Apical Na^+ conductance was measured as reported by others (Baines *et al.* 2001) with some modifications. Initially the cells were bathed in a low Na^+ solution (mM: Na^+ (11.5), K^+ (136.4), Cl^- (10.3), gluconate (122), HCO_3^- (25), Mg^{2+} (1.2), $H_2PO_4^-$ (1.2), Ca^{2+} (5.4)) in an Ussing chamber under short circuit conditions. After allowing the monolayers to stabilize for approximately 10 min, nystatin to a final concentration of 75 μM was added to the basolateral side. This concentration has previously been shown to fully permeabilize the cell membrane (Lewis *et al.* 1977) and application of higher concentrations of nystatin did not lead to a further increase in I_{sc} (data not shown). A few minutes later, the apical solution was replaced so that the final concentration of ions would be (mM): Na^+ (55), K^+ (92.9), Cl^- (10.3), Mg^{2+} (1.2), $H_2PO_4^-$ (1.2), Ca^{2+} (5.4), HCO_3^- (25) and gluconate (123.3). Simultaneously, the basolateral solution was changed to the final concentration of (mM): Na^+ (11.5), K^+ (92.9), Mg^{2+} (1.2), Ca^{2+} (5.4), NMDG (43.5), gluconate (123.3), Cl^- (10.3), HCO_3^- (25), $H_2PO_4^-$ (1.2). The amiloride-sensitive and amiloride-insensitive apical conductance values were calculated by dividing either the difference between baseline and post-amiloride (apical application of 0.1 mM) I_{sc} or the post-amiloride I_{sc} by the value of the calculated Nernst potential at 55 mM Na^+ (41.8 mV).

Northern analyses

RNA was extracted from DLE monolayers grown on permeant supports using 4 ml of TRIzol (Gibco BRL), re-dissolved in dimethyl pyrocarbonate (Sigma Aldrich)-treated water and size fractionated (20 μg) on a 1 % agarose–Mops–2 % formaldehyde gel. Following transfer to Hybond N^+ nylon membranes (Amersham Pharmacia Biotech, Baie d'Urfé, Quebec, Canada), RNA was UV cross-linked and hybridized with ^{32}P random primed cDNA probes. These cDNA probes included α-rENaC, β-rENaC, γ-rENaC cDNA probes (bp 74–403 for α-rENaC, 2025–2401 for β-rENaC and 2161–2520 for γ-rENaC) or rat α_1-ATPase, and β_1-ATPase (full length). All membranes were hybridized in Expresshyb solution (Clontech, Palo Alto, CA, USA). After washing in 0.1 × sodium citrate–NaCl (SSC) + 0.1 % sodium dodecyl sulfate (SDS) at 50 °C for 1 h, the blots were exposed to autoradiography film at −80 °C. Autoradiographic bands were quantified using an Agfa (Duoscan) scanner and the National Institutes of Health (NIH) Scion Image v1.6 quantification program. The mRNA levels were normalized to 18S ribosomal RNA content by hybridizing the blots with a full length mouse 18S ribosomal RNA ^{32}P random primed cDNA probe (American Type Culture Collection, Rockville, MD, USA).

Western analyses

Total protein was extracted from DLE on ice using a cold lysis buffer containing: 50 mM Hepes, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 % glycerol, 1 % Triton X, pH 7.5 in the presence of protease inhibitors (2 μg ml^{-1} aprotinin, 2 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A, 2 μg ml^{-1} antipain, 100 μg ml^{-1} phenylmethyl sulfonyl fluoride, 10 mM benzamidine and 1 mM EDTA). After extraction, the total protein content of the lysates was measured using Bio-Rad protein determination dye (Bio-Rad, Mississauga, Ontario, Canada). Fifty micrograms of total protein (unboiled) was loaded on standard 7.5 or 10 % acrylamide denaturing gel and size fractionated using conventional electrophoresis methods. The proteins were then transferred to a nitrocellulose membrane (Bio-Rad) using the Bio-Rad Western transfer system and standard protocols. After the membranes were blocked with PBS plus 5 % non-fat milk for 30 min, they were incubated in either 1:500 dilution (in 3 % bovine serum albumin (Sigma Aldrich) in PBS plus 0.05 % Tween 20) of anti-rat α_1-ATPase antibody (MCK1 monoclonal, generously provided by Dr K. J. Sweadner, Harvard University, Boston, MA, USA), or 1:1000 dilution (in 5 % non-fat milk in PBS plus 0.05 % Tween 20) of anti-rat β_1-Na–K^+ ATPase (Upstate Technology, Lake Placid, NY, USA) for 1 h. After washing with PBS + 0.05 % Tween 20, the membranes were exposed to either 1:10 000 dilution (in 5 % non-fat milk in PBS plus 0.05 % Tween 20) of horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody or to 1:20 000 dilution (in 5 % non-fat milk in PBS plus 0.05 % Tween 20) of horseradish peroxidase conjugated goat anti rabbit IgG antibody (Roche Diagnostics, Laval, Quebec, Canada). After blotting with α_1- and β_1-Na$^+$–K$^+$ ATPase, blots were reprobed initially with 1:10 000 dilution (in 5 % non-fat milk in PBS plus 0.05 % Tween 20) of anti-β-actin monoclonal AC-15 antibody (clone AC-15, Sigma-Aldrich) and then with 1:20 000 dilution (in 5 % non-fat milk in PBS plus 0.05 % Tween 20) of horseradish peroxidase conjugated rabbit anti-mouse IgG antibody. We used ECL chemiluminescence kit (Amersham Pharmacia Biotech) for final detection of the protein bands.

Fetal mice studies

For the majority of experiments, fetuses from timed pregnant wild-type Bl6 mice were studied. In some experiments Bl6 mice that were heterozygous for the α-ENaC knockout mutation (Hummler *et al.* 1996) were bred to obtain fetuses that were homozygous for the α-ENaC knockout mutation. Fetal lungs and trachea from 34 fetuses derived from six litters were studied. Mice were killed by exposing them to CO_2 for approximately 3 min.

Fetal tracheal studies

Seventeen- and eighteen-day fetal tracheas were explanted into a collagen gel and cultured in Ham's F12 plus 10 % FBS at 37 °C in a 95 % air–5 % CO_2 environment until they formed cysts (4–5 days) as described previously (Gillie *et al.* 2001). Culture medium was changed every 48 h. After 5 days in culture, the PD was measured across the wall of explants that formed liquid-filled cysts as previously described (Gillie *et al.* 2001). The cysts were impaled first with an electrode to determine basal PD, and then with a micropipette containing amiloride (10^{-2} M; Sigma, MO, USA). When the PD reached a stable value, amiloride was injected into the cyst lumen (final concentration 10^{-4} M) to inhibit amiloride-sensitive Na^+ channels. After the PD had stabilized, terbutaline sulfate (final concentration 3×10^{-5} M) was added to the bath to stimulate cAMP-sensitive Cl^- secretion.

Fetal distal lung liquid secretion/absorption studies

Distal lung fragments containing acinar and terminal bronchiolar epithelium were dissected from fetal lung and cultured to form liquid-filled cystic explants as described previously (Gillie *et al.* 2001). In brief, distal lung fragments containing acinar and terminal bronchiolar structures were dissected from lungs and placed on clear, permeable Falcon Transwell cell culture inserts (Becton-Dickinson, NJ, USA) in a culture dish containing a 1:1 F12–DMEM solution supplemented with bovine serum albumin (1 μg ml^{-1}). Each lung generated multiple explants for study. On average, four explants were placed on a single culture membrane. Three or four culture membranes from each lung were studied, depending on the number of experimental conditions (control, EF, heat-treated EF and plasma). Explants were cultured at 37 °C in a 95 % air–5 % CO_2 environment for the 3 day duration of the studies. After 24 h, the bathing medium was supplemented with normal saline, EF, heat-treated EF or plasma at 50 % concentration. Photographs were taken of the cultures at t = 0, 24 and 48 h and analysed with Metamorph software (FR Chemical, Albany, NY, USA) to determine change in cross-sectional area over time. Results of changes in explant size were pooled for each experimental group. For histology studies, explants were fixed in Omnifix (Universal Imaging Corp., Downington, PA, USA) and embedded in epoxy resin. Ultra thin sections were stained with haematoxylin and eosin for light microscopy. Sections from three control and EF-treated explants were photographed.

Data analysis

Student's t test, analysis of variance followed by the Tukey-Kramer multiple comparisons test or multiple t test with Bonferroni correction were used to examine significant differences (P values < 0.05) between experimental groups. Data are expressed as means ± standard error of the mean (S.E.M.).

RESULTS

Effect of EF on bioelectric properties of DLE

Exposure to rat EF for 24 h almost doubled the DLE's baseline I_{sc} relative to control medium cells with a nearly 4-fold increase in amiloride-insensitive I_{sc} (Fig. 1). The EF had a significant but biologically trivial effect on transepithelial R (EF = 1707 ± 46 Ω cm^2 *vs.* control

Table 1. Effect of rat bronchoalveolar lavage (BAL) fluid, pulmonary oedema fluid (EF), serum and plasma on ion transport by DLE

Group (n)	I_{sc} (μA cm^{-2}) Baseline	I_{sc} (μA cm^{-2}) Post-amiloride *
Control (106)	3.6 ± 0.09	1.0 ± 0.03
BAL (8)	3.2 ± 0.08	1.5 ± 0.05
EF (105)	6.7 ± 0.15	3.9 ± 0.06 †
Serum (12)	6.4 ± 0.35	1.5 ± 0.16
Plasma (5)	5.0 ± 1.09	0.6 ± 0.03

Values are means ± S.E.M. * Significant ($P < 0.05$) difference between post-amiloride and baseline in all groups. † Significant ($P < 0.05$) difference from corresponding control monolayers run concurrently.

medium = 2055 ± 70 Ω cm^2, $n = 105$, $P < 0.05$). None of BAL (obtained by lavaging normal rat lungs with DMEM alone), rat serum, or plasma (prepared following aortic clamping and EF collection from the same animals from which EF was harvested) increased the amiloride-insensitive I_{sc} of DLE monolayers (Table 1). However, serum- and plasma-exposed DLE had higher amiloride-sensitive I_{sc} than the control medium (Table 1). The EF-induced increase in total I_{sc} resulted from an increase in the amiloride-insensitive component as the amiloride-sensitive I_{sc} was similar to medium-exposed DLE (Fig. 1). The pulmonary EF-induced increase in amiloride-insensitive I_{sc} was both time (Fig. 2*A*), and dose dependent (Fig. 2*B*).

Additional experiments assessed whether other ion transport inhibitors could abrogate the EF's ability to induce amiloride-insensitive I_{sc}. Although several Cl^- and Na^+ transport inhibitors had a statistically significant effect on the amiloride-insensitive I_{sc}, none of these inhibitors could inhibit the majority of the oedema-induced amiloride-insensitive current (Table 2). Addition of the CNGC inhibitor diltiazem to the apical membrane of DLE monolayers led to a decrease in amiloride-insensitive I_{sc} (Table 2). Basolateral $BaCl_2$ also significantly abrogated oedema-induced amiloride-insensitive I_{sc} (Table 2). Addition of $BaCl_2$ to the apical side of DLE had no effect (data not shown). Combining several different Cl^- transport inhibitors, combining different Na^+ transport inhibitors or studying DLE in Ussing chambers containing HCO_3^--free medium could not completely block the induced amiloride-insensitive I_{sc} (data not shown). The largest effect was seen by combining niflumic acid and diltiazem, which reduced amiloride-insensitive I_{sc} by approximately 50 % (data not shown).

EF-induced I_{sc} is not abrogated by antioxidants

It has been shown previously that the amiloride-insensitive I_{sc} induced in DLE by-products from activated macrophages (Compeau *et al.* 1994) could be abrogated by antioxidants (Dickie *et al.* 2000). Therefore, we conducted additional experiments where we added either EUK-8 (30 μM) or *N*-acetyl cysteine (NAC, 20 mM) to medium or EF bathing the DLE ($n = 5$ each group). In EF-exposed DLE, EUK-8 significantly ($P < 0.05$) abrogated the increase in amiloride-insensitive I_{sc} but the extent of inhibition was immaterial (1.84 ± 0.1 *vs.* 2.28 ± 0.1 μA cm^{-2} in the control medium group). NAC failed to abrogate EF's effect on amiloride-sensitive I_{sc} (2.84 ± 0.07 μA cm^{-2}).

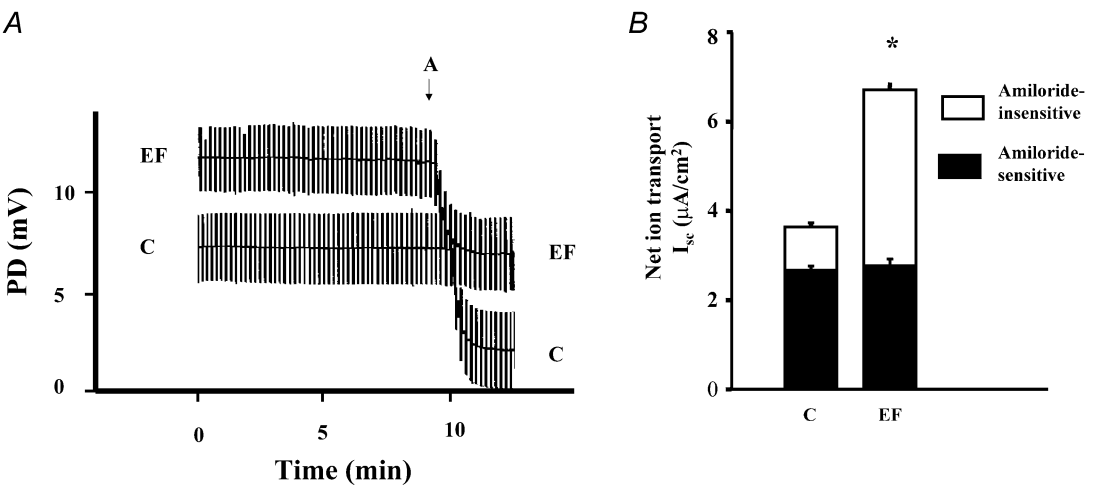

Figure 1. Exposure of primary cultures of rat distal lung epithelia (DLE) to pulmonary oedema fluid from rats in acute heart failure increased their amiloride-insensitive, but not amiloride-sensitive, ion transport

A, original trace of potential difference (PD) measurements before and after application of amiloride for medium (C) or oedema fluid (EF)-treated DLE. *B*, amiloride-sensitive and -insensitive I_{sc} in medium and EF-treated monolayers (* $P < 0.05$ between control medium (C) and EF-exposed DLE amiloride-insensitive current; I_{sc}, short circuit current; A, amiloride; $n = 105$).

Table 2. Effect of various blockers on DLE amiloride-insensitive I_{sc}

	I_{sc} (μA cm^{-2})			
	Media		Oedema fluid	
Blocker (Side, *n*, concentration)	Pre-inhibitor †	Post-inhibitor	Pre-inhibitor †	Post-inhibitor
Amiloride (AP, 0.1 mM)	3.6 ± 0.09	1.0 ± 0.03	6.7 ± 0.15	3.9 ± 0.06
Bumetanide (BL, 3, 0.1 mM)	1.0 ± 0.16	1.0 ± 0.21	3.7 ± 0.08	3.5 ± 0.06
DPAC (AP, 4, 1 mM)	1.0 ± 0.09	0.9 ± 0.07	3.6 ± 0.13	3.3 ± 0.13
Niflumic acid (AP, 4, 0.5 mM)	1.0 ± 0.08	0.9 ± 0.07	4.1 ± 0.33	3.0 ± 0.36
NPPB (AP, 4, 0.5 mM)	0.9 ± 0.04	0.8 ± 0.04	3.9 ± 0.24	3.1 ± 0.15
Glibenclamide (AP, 4, 0.5 mM)	0.8 ± 0.07	0.7 ± 0.07	3.9 ± 0.17	3.4 ± 0.16
L-*cis*-Diltiazem (AP, 8, 0.3 mM)	0.8 ± 0.05	0.5 ± 0.04	3.9 ± 0.08	2.1 ± 0.07 *
$CdCl_2$ (AP, 4, 1 mM)	0.9 ± 0.06	0.6 ± 0.13	4.3 ± 0.40	3.9 ± 0.40
Phloridzin (AP, 6, 1 mM)	1.0 ± 0.09	1.0 ± 0.10	1.6 ± 0.18	1.5 ± 0.13
$BaCl_2$ (BL, 4–6, 3 mM)	1.2 ± 0.13	0.6 ± 0.12	4.8 ± 0.38	1.9 ± 0.02 *
Ouabain (BL, 5–6, 1 mM)§	4.23 ± 0.09	1.40 ± 0.05	9.76 ± 0.18	2.78 ± 0.29
Flufenamic acid (AP, 7–8, 50 μM)‡	1.0 ± 0.21	0.7 ± 0.10	3.5 ± 0.06	2.9 ± 0.06 *

Values are means ± S.E.M. * Significant ($P < 0.05$) difference in the decrement in amiloride-insensitive I_{sc} arising from this inhibitor's use in the medium and oedema fluid groups. † Measured after addition of amiloride (10^{-4} M) to the apical membrane of the DLE monolayers. ‡ Administered after the application of amiloride (10^{-4} M) and bumetanide (10^{-4} M) to the apical and basolateral membranes, respectively (Ding *et al.* 1998). AP, apical; BL, basolateral. Ouabain was added after application of 50 μM to the apical membrane (amiloride was not added to this group), § $P < 0.05$ for comparison of magnitude of change after application of ouabain between control medium and oedema fluid group.

EF alters the expression of Na^+–K^+ ATPase in DLE monolayers and induces Na^+–K^+ ATPase activity

Although mRNA levels of the α_1 and β_1 subunits were unchanged (Fig. 3, n.s.) both α_1 and β_1 Na^+–K^+ ATPase protein contents (Fig. 4) increased when DLE were cultured in EF. This latter was consistent with the increase ($P < 0.05$) in ouabain-sensitive I_{sc} in the EF-exposed (6.9 ± 0.92) relative to control (4.0 ± 0.49 μA cm^{-2}) apically permeabilized DLE monolayers ($n = 5$).

EF does not induce ENaC mRNA expression

The EF's induction of an I_{sc} that was insensitive to amiloride suggested that the effect was independent of ENaC (Canessa *et al.* 1993, 1994; Lingueglia *et al.* 1993). Consistent with this speculation, α- and β-ENaC mRNA levels were unchanged and there was a statistically significant decrease in γ-ENaC mRNA levels (Fig. 5). An ENaC-independent mechanism is further supported by the finding that EF increased amiloride-insensitive but not amiloride-sensitive DLE apical membrane Na^+ conductance (Fig. 6).

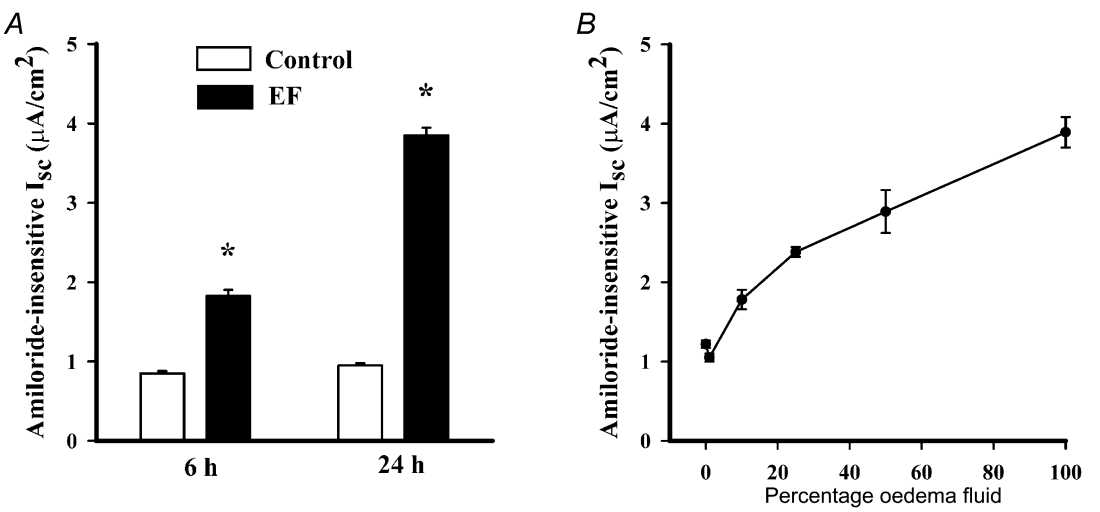

Figure 2. Induction of DLE's amiloride-insensitive I_{sc} by oedema fluid

Induction of DLE's amiloride-insensitive I_{sc} by oedema fluid (EF) was both time (*A*, $n = 4$) and dose (*B*, $n = 8$–12) dependent (I_{sc}, short circuit current, * $P < 0.05$).

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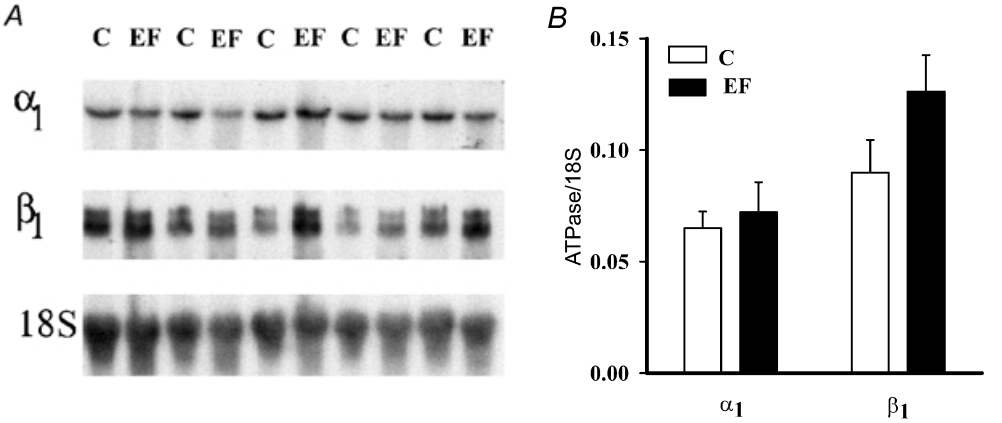

Figure 3. The mRNA concentrations of the α1 and β1 subunits of Na⁺–K⁺ ATPase

The mRNA concentrations of the α_1 and β_1 subunits of Na^+–K^+ ATPase were similar (n.s.) in control medium (C)- and pulmonary oedema fluid (EF)-exposed DLE. Results in *B* are normalized to 18S ribosomal RNA ($n = 5$, n.s. between control medium and EF-exposed DLE).

Induction of ion transport by EF is abrogated by heat and protease treatment

To determine if the effect of the EF was dependent upon the integrity of proteins contained within the fluid we tested the effects of heat- or trypsin-treated EF on DLE. As illustrated in Fig. 7 both these interventions abrogated the EF's ability to induce amiloride-insensitive I_{sc} in the DLE suggesting that the active compound(s) within the EF were proteins or required intact protein function.

EF induces amiloride-insensitive fluid absorption in murine lung

As our bioelectric measurements of DLE exposed to pulmonary EF could not determine whether the EF induced an amiloride-insensitive Na^+ fluid absorption or a

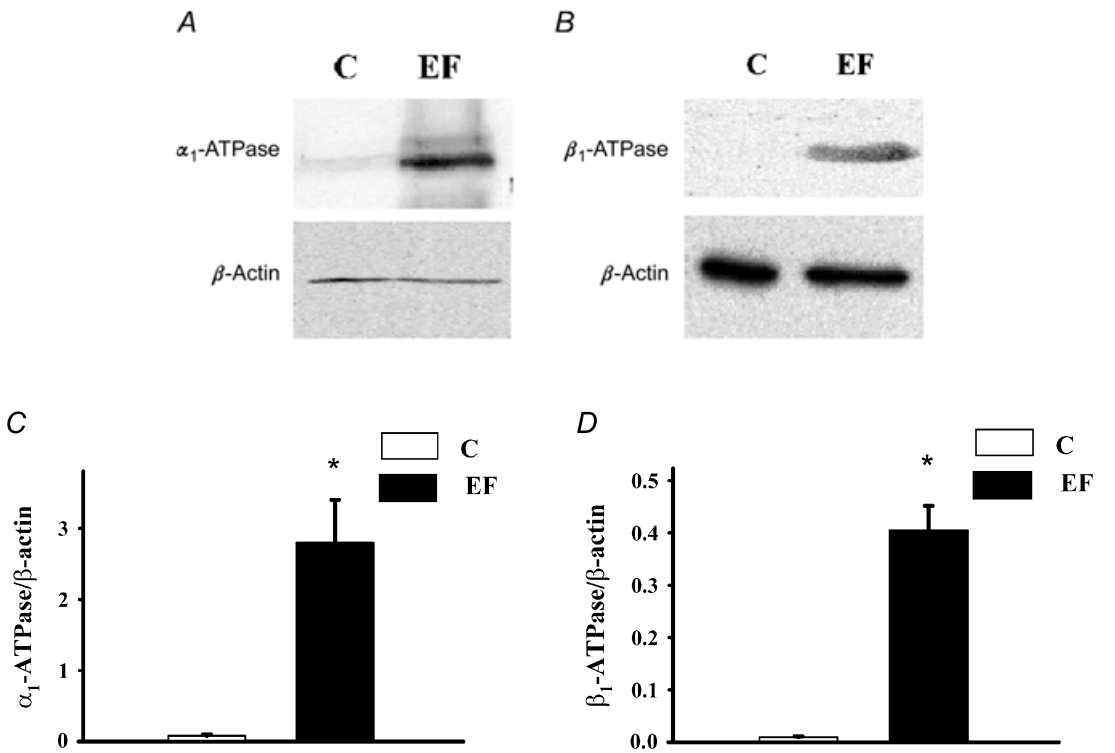

Figure 4. α_1 and β_1 Na^+–K^+ ATPase protein contents

The α_1 and β_1 Na^+–K^+ ATPase protein contents were increased (* $P < 0.05$ between control medium and EF-exposed DLE) in Western blots of DLE cultured in pulmonary oedema fluid relative to control monolayers. *A* and *B*, representative blots for α_1 (~100 kDa) and β_1 (~50 kDa) Na^+–K^+ ATPase Western blots reprobed with β-actin (~40 kDa); *C* and *D*, quantitative measurements of these blots normalized to β-actin levels ($n = 3$–4; C, control medium; EF, oedema fluid).

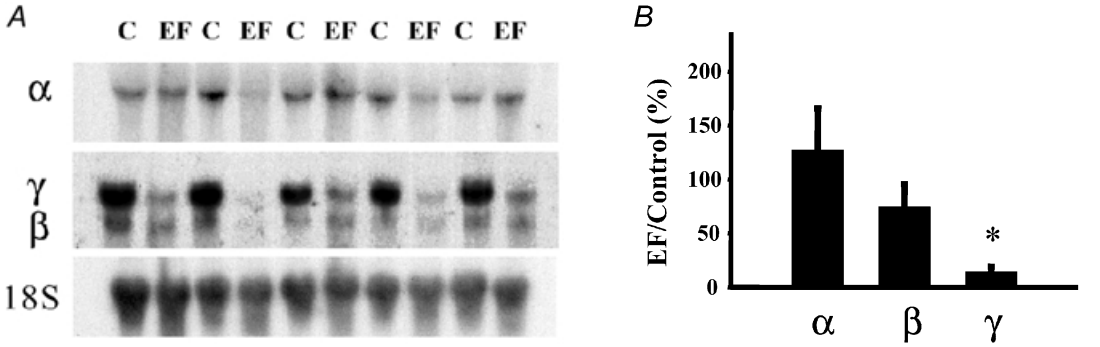

Figure 5. ENaC mRNA levels for oedema-exposed DLE

When DLE were exposed to oedema fluid, the α- and β-ENaC mRNA levels were unchanged relative to control medium (*B*, $n = 5$, n.s.) whereas γ-ENaC mRNA levels decreased (* $P < 0.05$ *vs.* α- and β-ENaC). Note, that in rat DLE the relative amounts of ENaC subunit mRNA are normally $\alpha > \gamma > \beta$. However, the different relative densities of the bands illustrated in *A* reflect different specific activities of the probe and different film exposure times (C, control medium; EF, oedema fluid).

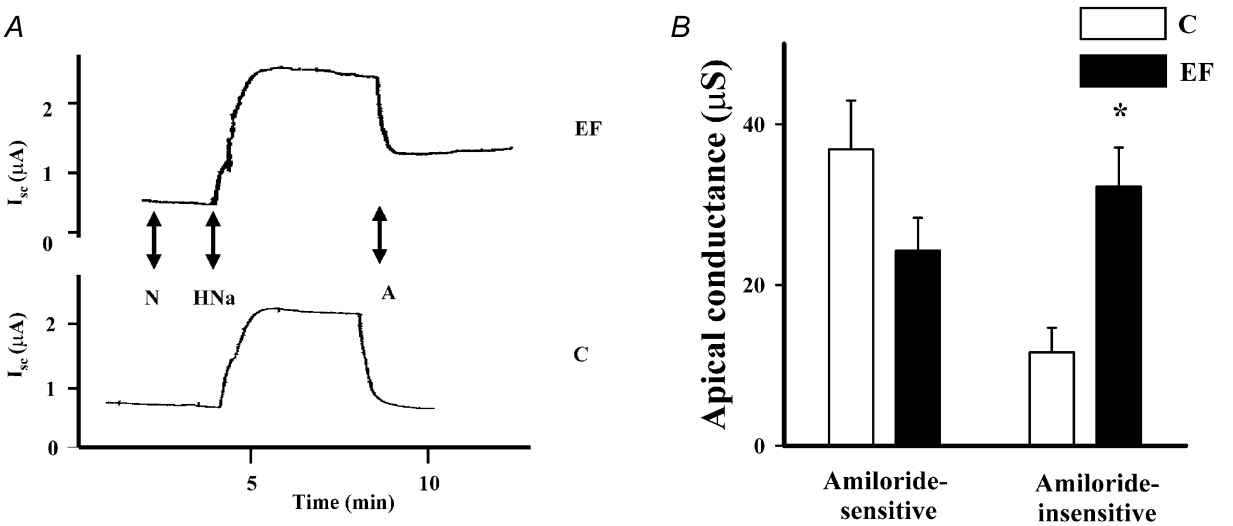

Figure 6. The amiloride-insensitive but not amiloride-sensitive apical membrane conductance for Na^+ was increased in DLE exposed to oedema fluid

A, representative trace; *B*, quantitative summary. C, control medium; EF, oedema fluid; $n = 8$, * $P < 0.05$ between control medium and EF-exposed DLE, S, Siemens; N, nystatin; HNa, high [Na^+]; A, amiloride, 10^{-4} M to apical side.

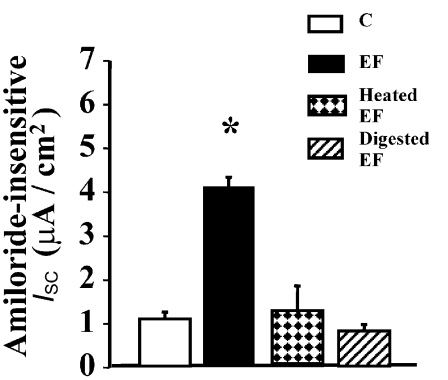

Figure 7. Trypsin digestion or heat treatment of the EF abrogates its ability to increase amiloride-insensitive I_{sc} in DLE

C, control medium; EF, oedema fluid, * $P < 0.05$ *vs.* other groups, $n = 5$–7.

bumetanide-insensitive Cl^- fluid secretion we performed additional experiments in late gestation fetal mice. We first determined that EF could induce changes in the murine respiratory epithelium's bioelectric properties. Consistent with our findings in rat DLE monolayers, micropuncture experiments showed that EF induced an amiloride- and bumetanide-insensitive PD in murine tracheal cysts (Fig. 8*A* and *B*). We then carried out studies to determine the effect of EF on liquid flow across the epithelium of mature fetal murine lungs. Cultured explants of fetal murine lung, which normally expand due to liquid secretion (cyst expansion from 24 to 48 h was 26.5 ± 1.5 %), decreased in size when exposed to EF (cyst contraction 31.9 ± 2.2 %). Sections of explants examined after fixation indicated that contraction of EF-treated explants had occurred as a result of absorption of liquid from the explant lumen (Fig. 8*C* and *D*). The absorptive effect was not seen when the explants were exposed to plasma (cyst expansion 8.2 ± 4.5 %, $n = 34$ explants) or to EF that had been heat-treated (cyst expansion 12.6 ± 2.6 %, $n = 32$ explants) (Fig. 8*D*). This suggested that the active compound(s) inducing the liquid absorptive response were proteins contained within the EF or were dependent upon protein integrity. To determine whether the EF's induction of an amiloride-insensitive liquid absorption in the distal lung was dependent upon ENaC, we carried out comparable studies with distal lung explants from α-ENaC-deficient mice. The liquid transport responses to medium and EF

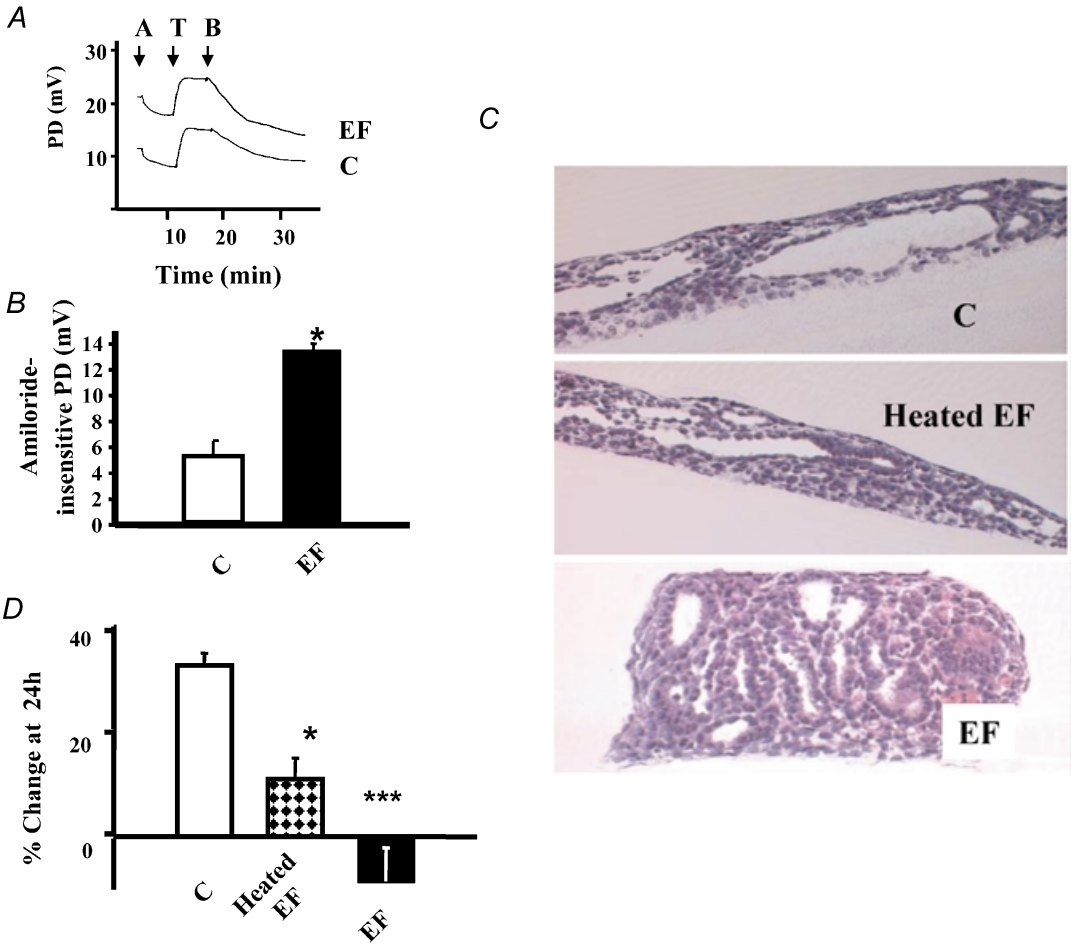

Figure 8. Oedema fluid induced amiloride-insensitive PD in murine tracheal cysts and abrogated liquid secretion in fetal murine explants

An amiloride-insensitive PD was induced in murine tracheal cysts when they were exposed to oedema fluid, but not when exposed to control medium. *A*, representative trace; *B*, summary of PD measurements. Bumetanide could not block this oedema fluid-induced PD (A, amiloride; B, bumetanide; T, terbutaline; summary for bumetanide results not shown). Consistent with these observations, oedema fluid induced a fluid absorptive response in distal lung explants from mature fetal murine lungs (*C* and *D*). *C* shows cross-section of wild-type explants exposed to control solution (DMEM–F12), heated 50 % EF or 50 % oedema fluid in DMEM–F12 for 24 h. The absorptive effect of EF was abrogated by prior heat treatment of the oedema fluid, and not seen in explants treated with 50 % plasma (data not shown). * $P < 0.01$ *vs.* control medium. EF, oedema fluid; C, control medium.

observed in fetal distal lung explants from homozygous α-ENaC deficient mice (α-ENaC−/−) were the same as those observed in explants from heterozygote and wild-type litter mate controls (medium, cyst expansion over 24 h: controls +23.4 ± 0.8 %, n = 18, α-ENaC−/− +26.1 ± 1.8 %, n = 7; EF, cyst contraction over 24 h: controls −31.9 ± 0.5 %, n = 25, α-ENaC−/− −28.8 ± 1.3 %, n = 6).

Together, the DLE monolayer and murine lung explant experiments indicate that EF induces an α-ENaC-independent apical membrane Na^+ conductance and liquid absorption in distal lung epithelia, accompanied by concurrent increase in Na^+–K^+ ATPase activity.

DISCUSSION

This study provides the first demonstration that pulmonary oedema fluid itself alters ion transport in distal lung epithelia. The effect of the oedema is both time and dose dependent and the causative factors within the EF either are proteins or require intact proteins to exert their effect. These factors induce a novel α-ENaC-independent and amiloride-insensitive apical membrane Na^+ conductance and fluid absorption in the epithelium of the distal lung regions.

The apical membrane entry of Na^+ is the rate-limiting step in epithelial Na^+ transport under usual conditions. However, the apical membrane entry pathway varies between tissues and in the case of the lung there has been controversy concerning the existence of multiple epithelial Na^+ pathways in lung epithelia. The initial patch clamp studies of DLE grown in primary culture suggested that the predominant Na^+ conductive channel had a ~25 pS conductance, that it was amiloride-sensitive and that it did not discriminate between Na^+ and K^+ (Orser *et al.* 1991). These biophysical characteristics are considerably different from the amiloride-sensitive, 4–8 pS highly Na^+ selective Na^+ channel present in aldosterone-treated amphibian A6 renal epithelial cell lines (Eaton *et al.* 1995) or when α (Canessa *et al.* 1993; Lingueglia *et al.* 1993), β and γ subunits (Canessa *et al.* 1994) of the amiloride-sensitive epithelial Na^+ channel (ENaC) are expressed in oocytes. Since α, β and γ subunits are expressed in distal lung epithelium (O'Brodovich *et al.* 1993; Tchepichev *et al.* 1995) and 4–8 pS highly Na^+ selective Na^+ channels have been identified in lung epithelium (Voilley *et al.* 1994), ENaC is present and functional within at least some regions of the lung. However, genetic experiments provide support for non-ENaC Na^+ conductive pathways within the lung. Specifically, although the α-ENaC subunit is essential for murine perinatal lung water clearance (Hummler *et al.* 1996), newborn mice lacking either the β or γ subunits have normal or near normal lung water clearance (Barker *et al.* 1998; McDonald *et al.* 1999). In addition, patients with pseudohypoaldosteronism, who are the 'human α-ENaC knockout', do not have a history of significant respiratory distress at birth (Chang *et al.* 1996). The existence of α-ENaC-independent Na^+ transport pathways within the lung is strongly supported by our present experiments where EF did not increase ENaC mRNA or the DLE's amiloride-sensitive apical membrane conductance, yet induced fluid absorption in distal lung explants from α-ENaC-deficient mice.

Our present experiments are consistent with previous studies that show the presence of amiloride-insensitive Na^+ transport pathways within the distal regions of the lung. Studies in single pass perfused lungs revealed amiloride-insensitive $^{22}Na^+$ and airspace fluid clearance (O'Brodovich *et al.* 1997) and there is amiloride-insensitive $^{22}Na^+$ transport in acutely isolated bovine airway (Langridge-Smith, 1986) and rabbit nasal epithelium (Ropke *et al.* 1996). DLE grown in primary culture have variable amounts of Na^+-dependent, amiloride-insensitive I_{sc} (O'Brodovich *et al.* 1990) and human nasal epithelia have amiloride-insensitive Na^+ conductive channels (Chinet *et al.* 1993). Previous work has also demonstrated that these amiloride-insensitive pathways can be modulated *in vivo* and *in vitro*. For example, dopamine increases amiloride-insensitive fluid clearance in isolated rat lungs (Barnard *et al.* 1997). When DLE are grown in culture with activated macrophages, there is a downregulation of the amount of amiloride-sensitive Na^+ transport (Compeau *et al.* 1994) and ENaC mRNA levels (Dickie *et al.* 2000) with a concomitant increase in amiloride- and bumetanide-insensitive I_{sc} (Compeau *et al.* 1994; Dickie *et al.* 2000) that is partially sensitive to flufenamic acid (Ding *et al.* 1998), a blocker of non-selective cation and Ca^{2+}-activated Cl^- channels. It is unlikely that our present results can be explained by activated macrophages since flufenamic acid had minimal effects on the EF exposed DLE (Table 2), the BAL control experiments did not augment amiloride-insensitive I_{sc} (Table 1), and the anti-oxidants NAC and EUK-8 could not efficiently abrogate the effect of the EF (see Results).

Interestingly, plasma- and serum-exposed DLE had higher amiloride-sensitive I_{sc} than the medium-treated controls. Since EF is mainly composed of a plasma filtrate, it is unclear why amiloride-sensitive I_{sc} was not similarly induced by EF. Possible explanations are that components present in EF derived from the interstitial or alveolar space somehow counteract the ability of plasma to increase amiloride-sensitive I_{sc} in DLE, or that the components in plasma which increase amiloride-sensitive I_{sc} do not cross into the airspace during the formation of hydrostatic oedema.

Our results also point to increased protein content and activity of Na^+–K^+ ATPase by EF without an observed increase in expression of mRNA for this protein. This

observation may be due to increased stability of this enzyme or more efficient translation of its mRNA.

This study indicates that the oedema-induced increase in amiloride-insensitive Na^+ transport is associated with an increased apical membrane amiloride-insensitive conductive pathway (Fig. 6). One potential explanation is that the oedema augmented the expression of the CNGC that has been identified within the lung and is relatively insensitive to amiloride (Ding *et al.* 1997). Expression of CNGC is known to be developmentally regulated as studies suggest that it plays a role in fluid clearance from the lungs of 6-month-old (Junor *et al.* 1999) but not 6-week-old lambs (Junor *et al.* 2000). Although CNGC does not appear to be active in the rat lung under resting conditions, diltiazam, a blocker of CNGCs, can block part of a cGMP analogue-stimulated increase in lung liquid clearance (Norlin *et al.* 2001). However, increased expression of CNGC is an unlikely explanation for increased amiloride-insensitive ion transport in DLE in the presence of EF as only a portion of the oedema-induced amiloride-insensitive I_{sc} was blockable with diltiazem (Table 2). In addition, our previous studies have shown that membrane-permeant analogues of cGMP do not increase I_{sc} in mature fetal DLE (O'Brodovich *et al.* 1992).

Although our genetic experiments indicate that α-ENaC is not required for this phenomenon, we cannot rule out the unlikely possibility of a β- and γ-ENaC containing a Na^+ channel that has a non-functional amiloride binding site. Our results also show that the induction of amiloride-insensitive I_{sc} by EF is associated with a profound reduction in γ-rENaC mRNA levels. Therefore, one may speculate that a reduction in γ-rENaC expression could have altered amiloride sensitivity of ENaC in DLE, for example due to increased α and β expression on the cell surface in an amiloride-insensitive conformation. Several observations argue against this hypothesis. Specifically, as discussed above, α-ENaC is not required for the response to oedema fluid. In addition, expression of ENaC subunits individually or pairwise in heterologous systems leads to increased Na^+ current which is amiloride sensitive. We do not believe an alteration in γ-ENaC expression would alter amiloride sensitivity of ENaC since the presence of all subunits of ENaC is essential for optimum ENaC function. Thus we speculate that the EF induces an unknown Na^+-permeable ion channel.

Our bioelectric studies of DLE monolayers demonstrated that EF increased net transepithelial ion transport. However, since the majority of the increased I_{sc} was insensitive to classic inhibitors of Na^+ and Cl^- transport (Table 2), these experiments could not determine if the induced I_{sc} reflected an increase in Na^+ transport with concomitant liquid absorption, or increased Cl^- transport with concomitant liquid secretion. Direct measurement of lung epithelial liquid transport rate is difficult. Approaches have included the use of bronchial xenografts (Zhang *et al.* 1996), capacitance measurements across cultured airway epithelium (Jiang *et al.* 1993), biofibres (Grubb *et al.* 1997) and fluorescent dye-based techniques (Phillips *et al.* 1999). However, each approach has its limitations. For example, the fluorescent dye-based technique (Phillips *et al.* 1999) requires sophisticated technology and both it and the biofibre technique (Grubb *et al.* 1997) are at their limits of sensitivity when measuring unidirectional fluid flux across tracheal epithelium which has an I_{sc} of approximately 40 μA cm^{-2}. Our DLE have an I_{sc} of only ~4 μA cm^{-2}. We therefore utilized an approach that relies upon morphometric measurements of the cross-sectional area of distal lung explants and has been useful in the study of fetal lung liquid secretion (Gillie *et al.* 2001). We have previously shown that changes in cyst size reflect changes in liquid volume of the distal lung explant (Barker & Gatzy, 1993). The small inhibition of cyst expansion rate that was seen after exposing explants to either plasma or heat-treated EF may be due to the oncotic pressure exerted by these proteins. By contrast, EF induced a clear liquid-absorptive, and hence presumably Na^+-absorptive, response in distal lung explants of mature wild-type and α-ENaC-deficient fetal mice. These results indicate that the perinatal lung epithelium can express a Na^+-permeable ion channel that is different from the typical ENaC consisting of α, β and γ subunits.

Our preliminary investigations, using trypsin and heat denaturation, indicate that the EF's ability to induce an amiloride-insensitive Na^+ and fluid absorption is dependent upon the integrity of proteins contained within the EF. The biological effect of the EF may arise from a protein or combination of proteins, a group of small molecules, or a supramolecular complex. For example, channel activating protease (CAP1), a 329 residue serine protease found in the cytoplasmic membrane of Na^+ transporting epithelia, can activate ENaC (Vallet *et al.* 1997) and potentially other ion channels. Alternatively, it is known that lipids or fatty acids, which can modulate ion transport (Fyfe *et al.* 1997), can also bind to plasma proteins.

In conclusion, EF induces an amiloride-insensitive, α-ENaC-independent apical pathway with concomitant increase in basolateral Na^+–K^+ ATPase activity in lung epithelia. Further studies will be required to identify the active compound(s) which, once identified, may be useful for treatment of pulmonary oedema.

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