Effects of cardiogenic edema fluid on ion and fluid transport in the adult lung

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1Canadian Institutes of Health Research Group in Lung Development and 2Physiology and Experimental Medicine, 3Genetics and Genome Biology of the Research Institute of the Hospital for Sick Children, and Departments of 4Paediatrics, 5Physiology, and 6Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada; and Departments of 7Physiology and 8Pediatrics, Emory University, Atlanta, Georgia

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Gandhi SG, Rafii B, Harris MS, Garces A, Mahuran D, Chen X-J, Bao H-F, Jain L, Eaton DC, Otulakowski G, O’Brodovich H. Effects of cardiogenic edema fluid on ion and fluid transport in the adult lung. Am J Physiol Lung Cell Mol Physiol 293: L651–L659, 2007. First published June 8, 2007; doi:10.1152/ajplung.00464.2006.—We have previously shown that cardiogenic pulmonary edema fluid (EF) increases Na+ and fluid transport by fetal distal lung epithelium (FDLE) (Rafii B, Gillie DJ, Sulowski C, Hannam V, Cheung T, Otulakowski G, Barker PM, and O’Brodovich H. J Physiol 544: 537–548, 2002). We now report the effect of EF on Na+ and fluid transport by the adult lung. We first studied primary cultures of adult type II (ATII) epithelium and found that overnight exposure to EF increased Na+ transport, while this effect was mainly due to factors other than catecholamines. Plasma did not stimulate Na+ transport in ATII. Purification of EF demonstrated that at least some agent(s) responsible for the amiloride-insensitive component resided within the globulin fraction. ATII exposed to globulins demonstrated a conversion of amiloride-sensitive short-circuit current (Isc) to amiloride-insensitive Isc with no increase in total Isc. Patch-clamp studies showed that ATII exposed to EF for 18 h had increased the number of highly selective Na+ channels in their apical membrane. In situ acute exposure to EF increased the open probability of Na+-permeant ion channels in ATII within rat lung slices. EF did increase, by amiloride-sensitive pathways, the alveolar fluid clearance from the lungs of adult rats. We conclude that cardiogenic EF increases Na+ transport by adult lung epithelia in primary cell culture, in situ and in vivo.

PULMONARY EDEMA CAN OCCUR as a result of an increased transvascular hydrostatic pressure gradient, as seen in congestive heart failure (CHF), or increased permeability of the alveolar-capillary membrane to solutes, as occurs in adult respiratory distress syndrome (ARDS). Previous studies have shown that 75% of patients with CHF-induced pulmonary edema have demonstrable alveolar fluid clearance (AFC), and of these, only ~40% are able to achieve a maximal rate of AFC (38). Furthermore, patients with impaired AFC have significantly higher mortality rates and require longer periods of assisted ventilation compared with patients with maximal fluid clearance rates (40). Active fluid transport is therefore important for favorable patient outcomes, and methods of enhancing fluid clearance should have therapeutic benefits.

In the distal units of the lung, fluid transport occurs primarily through the active transport of Na+ through epithelial cation-permeant ion channels, with Cl− and water following (22, 23, 26). Normal mammalian lung epithelia have been shown to have both an amiloride-sensitive and an amiloride-insensitive component to their active Na+ transport both in vivo (e.g., Ref. 28) and in vitro (10, 17, 29). In vivo, this amiloride-insensitive component may represent up to 50% of the total AFC (23). The amiloride-insensitive epithelial Na+ channel (ENaC) is partially responsible for apical Na+ transport; however, recent studies have demonstrated the importance of other cation-permeant channels in the alveolar regions of the lung (18, 19).

Our laboratory has previously shown that exposure to rat cardiogenic pulmonary edema fluid (EF) results in increased ion transport by primary cultures of fetal distal lung epithelium (FDLE). Inhibitors of Cl− transporters or cyclic nucleotide-gated cation (CNGC) channels had limited ability to block the increased amiloride-insensitive short-circuit current in EF-exposed FDLE. The effect of the edema fluid was time and concentration dependent, and the causative factors were either proteins or required intact proteins to exert their effect (32).

Our previous work was done using primary cultures of FDLE as a perinatal model for ion transport. In this study, we focus on adult lung epithelium, using primary cultures of adult type II (ATII) epithelia, albeit recognizing that in the intact lung the vast majority of the alveolar air space is covered by alveolar type I (ATI) epithelia. To better understand how ion transport changed upon exposure to EF, serum globulins, and plasma, we carried out dose-response studies on monolayers of ATII, using Ussing chambers. We found that EF did alter ion transport in ATII, and furthermore, this effect was not simply due to catecholamines within the EF. We carried out patch-clamp studies on ATII that were grown in primary culture and on ATII in situ within lung slices from adult rats. We then completed direct measurements of fluid transport by primary cultures of ATII. Since the adult lung contains, in addition to ATII, several cell types that may contribute to Na+ and fluid transport (18, 19), we also tested the effects of EF and plasma by using healthy adult rats to observe the effects of these treatments on lung fluid clearance in vivo.

METHODS

Animals. All animal procedures were reviewed and approved by the Animal Care Committee of the Hospital for Sick Children ( SickKids)
and Institutional Animal Care/Use Committee guidelines of Emory University.

Materials. Tissue culture media and supplements were obtained from Invitrogen (Burlington, ON, Canada) for studies in Toronto. Unless specified otherwise, all other reagents and proteins were obtained from Sigma Chemical (Oakville, ON, Canada). The sources of reagents and materials for studies carried out at Emory University are described within the relevant sections.

Edema fluid preparation and fractionation. Cardiogenic pulmonary EF and plasma were obtained from rats according to protocols published previously (32). Briefly, 350- to 550-g male Sprague-Dawley rats were anesthetized using ketamine (80 mg/kg) and xylazine (8 mg/kg). A tracheostomy was performed, and assisted ventilation (fraction of inspired O2 (FiO2) = 1.0, tidal volume = 5 ml, rate = 38 breaths/min) was commenced. A thoracotomy was performed, the aorta was clamped for 30 s, and, after infusion of 15 ml/kg Ringer solution through the penile vein, EF was collected through the endotracheal catheter. The EF was centrifuged at 10,000 g for 30 min at 4°C. Plasma was obtained by centrifuging heparinized blood at 10,000 g for 30 min at 4°C before use.

Plasma and the EF supernatant were stored in aliquots at −85°C. EF was filter sterilized and diluted with Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal bovine serum (FBS) for Ussing chamber experiments. Catecholamine levels within the EF were measured using reverse phase HPLC with electrochemical detection. To determine the heat sensitivity of the active factors within EF, EF was heated at 80°C for 20 min and centrifuged at 10,000 g for 10 min at 4°C before use.

EF was fractionated using Cibacron blue dye affinity chromatography. Briefly, EF was desalted before application on an Affi-Gel blue F3GA column (Bio-Rad Laboratories, Mississauga, ON, Canada) and eluted with increasing concentrations of NaCl. Eluted fractions were sequentially dialyzed against PBS (0.1 M phosphate buffer, pH 7.2, containing 0.15 M NaCl) and DMEM, followed by filter sterilization before addition to cultured FDLE.

Fetal distal lung epithelium primary culture. FDLE from 20-day-gestation fetuses were isolated as previously described (30, 32). FDLE were seeded at 1 × 10^6 cells/cm^2 onto 0.4-μm pore size Snapwell cell culture inserts (Corning Costar, Cambridge, MA) for Ussing chamber studies or 24-mm Transwell inserts for determination of mRNA levels. All cells were grown as submersion cultures in DMEM (4.5 g/l glucose with 2 mM l-glutamine and 110 mg/l sodium pyruvate) supplemented with 10% FBS (Cansera, Rexdale, ON, Canada), 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate. 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 EF, plasma, or globulin (diluted with DMEM + 10% FBS), and bioelectric assessments were performed 18–24 h later.

ATII cell primary culture for Ussing chamber studies. ATII cells were isolated as previously described by others (13) with minor modifications as indicated within the text or legends. Briefly, 100- to 200-g male Sprague-Dawley rats were anesthetized using ketamine (80 mg/kg) and xylazine (8 mg/kg). Rats were ventilated through a tracheal tube (tidal volume = 2.0 ml/150 g, positive end-expiratory pressure = 1.0 cmH2O, rate = 38 breaths/min). A thoracotomy was performed, and heparin was injected into the right atrium. After exsanguination, the lungs were perfused through the pulmonary artery and bronchoalveolar lavage was performed. ATII cells were loosed with an elastase incubation (3 U/ml; Worthington, Lakewood, NJ) and bronchoalveolar lavage was preformed. ATII cells were loosened and bronchoalveolar lavage was performed. ATII cells were isolated, seeded onto Millipore permeable inserts following an additional 18- to 24-h incubation. We chose to grow ATII as submersions cultures for most experiments to better simulate the fluid-filled air space of an edematous lung.

Using chamber bioelectric measurements. Epithelial cells were studied in Ussing chambers at 37°C maintained under open-circuit conditions and then switched to short-circuit current with transepithelial potential difference, short-circuit current (Isc), and transepithelial resistances determined intermittently with voltage-current clamps (32). Addition of amiloride (final concentration = 10−4 M) to the apical side of the monolayers yielded amiloride-sensitive and -insensitive Isc.

Patch-clamp studies of primary cultures of ATII. At Emory University, the ATII were isolated, seeded onto Millipore permeable supports, and cultured for 24 h submerged in three parts Coon’s medication of Ham’s F-12 and seven parts Liebovitz’s L-15 with 1 μM dexamethasone. To better simulate the in vivo rat AFC studies (see Alveolar fluid clearance), the medium overlying the apical surface was then removed, and ATII were cultured with an apical air-liquid interface for an additional 24 h before experiments were carried out.

After basolateral exposure to EF for 18 h, the ATII were studied using cell-attached patch-clamp configurations at room temperature. Cell-attached bath and pipette solutions contained (in mM) 140 NaCl, 1 MgCl2, 1 CaCl2, 5 KCl, and 10 HEPES, adjusted to pH 7.4 with NaOH. Current-amplitude histograms were made from stable continuously recorded data, and the open and closed current levels were determined from least-squares-fitted Gaussian distributions. The number of channels (N) times single-channel open probability (P0) indicated patch channel activity. The total number of a patch’s functional channels (N) was estimated by observing the number of peaks in current-amplitude histograms constructed and, when possible, from long-record events to provide 95% confidence of the correct N value (21). The effect of EF was determined after 18-h exposure to this agent on the basolateral side.

Patch-clamp studies of ATII in situ. At Emory University, male Sprague-Dawley rats were maintained with access to standard rat diet and water ad libitum. At 8–12 wk of age, animals were anesthetized and killed in accordance with Institutional Animal Care/Use Committee guidelines. Following lung perfusion via the pulmonary artery with 75 ml of warm (35°C) PBS, warm 2% low-melting-point agarose in PBS was intratracheally instilled into the lungs to expand the air spaces and provide support for the tissue during the slicing process. Excised lungs were removed en bloc and chilled in cold PBS (4°C) to solidify the agarose, and a small block of tissue was separated from the largest lobe of the lung and mounted using surgical grade cyanoacrylate adhesive onto a VT1000S vibratome (Leica Microsystems, Bannockburn, IL). The vibratome blade was set to high-frequency vibration but advanced slowly through the continuously chilled tissue to make 275-μm-thick tissue slices. Tissue samples were placed in ice-cold DMEM/F-12 50:50 medium (containing 10% FBS, 2 mM l-glutamine, 1 μM Dex, 84 μM gentamycin, and 20 U/ml penicillin and streptomycin) until patch-clamp analysis was performed. Lung tissue was used within 4 h of the initial lung slicing.

To carry out the in situ patch-clamp studies, we rinsed the lung slices three times in recording solution (see immediately below) before patch clamping. Experiments were conducted at 22–23°C, and all patch recording solutions for the cell-attached configuration con-
tained (in mM) 140 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, and 10 HEPES at pH 7.4. Gigaohm seals were made between the tip of a fire-polished glass microelectrode (backfilled with patch solution) and the lung slice immersed in patch solution. An Axopatch 1-D amplifier (Molecular Devices, Sunnyvale, CA), interfaced through an analog-to-digital board to a personal computer, collected single-channel data. Channel currents were recorded at 2 Hz and subsequently filtered at 100 Hz with a low-pass Bessel filter. We used the product of the number of channels (N) times the single open probability (Pₒ) as a measure of channel activity within a patch. This product was calculated using FETCHAN and pCLAMP 6 software (Molecular Devices). We then calculated Pₒ by estimating the number of channels from the number of discrete levels observed in a patch.

**Fluid transport measurement.** Fluid transport was measured using methods similar to those previously published (16). Three days postseeding, ATII cultured on 6.5-mm Transwell permeable inserts were treated with EF diluted in DMEM + 10% FBS (total: 130 µl added above cells, 600 µl below) in the presence and absence of 100 µM amiloride added both apically and basolaterally. Fluid remaining above the cells was removed and weighed after 48 h. To correct for differences in densities between medium and EF, 130 µl of this medium was weighed and a corrected volume added was determined. Fluid transport was calculated from the difference between corrected volume added at time 0 and volume remaining 48 h later. Corrections were also made for fluid loss due to evaporation by using blank wells treated with the same volume of medium per surface area.

**Alveolar fluid clearance.** AFC measurements were carried out in anesthetized (80 mg/kg ketamine, 8 mg/kg xylazine) male Sprague-Dawley rats (317 ± 3.6 g) by instilling 0.5 ml of Ringer-lactate, EF, heat-treated EF, plasma, or bovine serum albumin (BSA; 18 g/dl) into each lung under fluoroscopic guidance. One day later, rats were reanesthetized (85 mg/kg pentobarbital sodium) and ventilated (Fₒ₂ = 1, tidal volume = 8 ml/kg, rate = 40–50 breaths/min) through an endotracheal tube. Blood pressure, heart rate, and blood gases were monitored via a carotid arterial line. After an initial 30-min period during which the animal was allowed to stabilize, 6 ml/kg of an isotonic FITC-dextran (>200 KD, 0.4 mg FITC-dextran/ml, 50 mg/ml BSA) Ringer solution (AFC solution) was instilled into one lung. Arterial partial pressure of CO₂ (Paco₂) was monitored throughout the experiment and maintained at 35–40 mm Hg by adjusting the ventilator rate. Osmolality of AFC solution was adjusted to that of plasma using concentrated saline. AFC (% of solution cleared per hour) was calculated from dextran concentrations at time 0 and 30 min (41) using the following formula: AFC = (1 − Cᵣ/Cᵢ) × 2, where Cᵢ and Cᵣ are the initial and final concentrations of dextran, respectively.

**Statistical analysis.*** Data are means ± SE. Student’s t-tests, using Bonferroni correction where required, or parametric analysis of variance, including Tukey’s test for post hoc analysis, were used to test for statistical significance (P < 0.05). In cases where the parametric assumptions of normality and/or equality of variance between the groups were not followed, appropriate nonparametric tests were used instead.

**RESULTS**

**EF increases ion transport by ATII in primary culture.** To evaluate the effects of EF in adult lung epithelium, we used primary cultures of ATII cells. EF treatment resulted in increased ion transport in ATII, and this effect was dose dependent (Fig. 1). Maximal effects on amiloride-insensitive current (AIC: 3.7 ± 0.3 µA/cm², n = 15, vs. 1.6 ± 0.1 µA/cm² in medium, n = 25, P < 0.001) and total Iₑ (8.4 ± 0.7 µA/cm², n = 15, vs. 5.8 ± 0.4 µA/cm² in medium, n = 25, P < 0.05) were seen at 25% EF. Amiloride-sensitive current (ASC) did not show any statistically significant changes; however, there was a trend observed in which ASC was increased by EF concentrations <25% EF and decreased with concentrations >25%. At a dose of 25% EF, heat treatment of EF inactivates its effect on AIC (1.6 ± 0.2 µA/cm² compared with 1.9 ± 0.2 µA/cm² in medium, n = 11).

*EF effects on Iₑ are not due to catecholamines in EF.* Although we determined that there were significant levels of catecholamines within the EF (3.9 nM norepinephrine, 2.6 nM epinephrine, and 1.1 nM dopamine in 100% EF), catecholamines could not account for EF-induced changes in bioelectric characteristics of ATII. When ATII were treated with catecholamines at a concentration similar to that found in 50% EF for a period of 20 h, no changes were observed in the AIC, ASC, or total Iₑ (Fig. 2).

The increase in AIC observed in EF-treated cells was not prevented by the addition of catecholamine receptor blockers [10 µM propranolol, 10 µM SCH-23390, 10 µM S(−)-sulpiride] when cells were exposed to either 50% EF (Fig. 2A) or 25% EF (Fig. 3A).

*ATII cultured at air-liquid interface respond to EF.* ATII cultured at the air-liquid interface responded to EF in a manner similar to submersion cultures. ATII at the air-liquid interface treated with 25% EF had significantly increased AIC and total Iₑ relative to controls (Fig. 3). The induction in AIC was not abrogated with the addition of catecholamine receptor blockers [10 µM propranolol, 10 µM SCH-23390, 10 µM S(−)-sulpiride] (Fig. 3A). Therefore, in contrast to submersion cultures, the trend toward increased ASC in response to EF was statistically significant in ATII cultured in air-liquid interface. This increase in ASC was prevented by catecholamine receptor blockers.

*Plasma effects differ from EF effects.* Since EF is a filtrate of plasma, we evaluated the effects on ATII cell ion transport of treatment with 0 to 25% rat plasma. We attempted to study ATII exposed to 50% rat plasma; however, high-resistance monolayers could not be maintained at that dosage. There was no statistically significant effect of plasma on Iₑ, Iₑ relative to controls (Fig. 3). The induction in AIC was not abrogated with the addition of catecholamine receptor blockers [10 µM propranolol, 10 µM SCH-23390, 10 µM S(−)-sulpiride] (Fig. 3A). However, in contrast to submersion cultures, the trend toward increased ASC in response to EF was statistically significant in ATII cultured at air-liquid interface. This increase in ASC was prevented by catecholamine receptor blockers.
Effects of EF components and serum proteins on AIC. In an attempt to separate active factors within EF, we chose to use Cibacron blue F3GA for initial fractionation of EF proteins to remove the very abundant protein albumin. This step would have facilitated a proteomics-based approach to identify candidate proteins for the AIC-stimulating effect by comparing the composition of EF with plasma in view of the latter’s inability to induce AIC in FDLE (32). However, separation of EF proteins using this approach showed that the activity copurified with albumin in the final, high-salt elution step (Fig. 5A).

Exposing FDLE to commercial preparations of rat serum albumin at a concentration comparable to the albumin concentration in EF showed that Cohn fraction V (96% pure), but not highly purified (99% pure), rat albumin stimulated AIC (Fig. 5B). BSA had no effect (data not shown). Highly purified rat albumin is derived from Cohn fraction V via removal of globulins and hydrophobic molecules (mainly free fatty acids).

The combination of 99% pure albumin and a Folch extract of hydrophobic molecules prepared from the Cohn fraction V albumin did not stimulate AIC (data not shown). In contrast, exposure of FDLE to Cohn fraction IV-4 globulins, which are...
when exposed to 50% EF for 18 h, there was a 1.4 ± 0.15-fold increase (*P < 0.01; Fig. 7A) in the apical membrane density of the highly Na⁺-selective channels (HSC) with no change in the density of nonselective cation channels (NSC). There was no significant change in single-channel current or the P₀ of the HSC, but there was a reduction in the P₀ of the NSC (*P < 0.013; Fig. 7B). Single-channel current did not change in either the HSC or the NSC after the 18-h exposure to 50% EF.

When EF was added to the solution bathing the in situ lung slice, there was an activation of the HSC in the apical membrane of the ATII (Fig. 7, C and D). Since this was a cell-attached patch, it suggests that the EF was capable of activating Na⁺ channel-activating signal transduction pathways within the ATII in situ. The effect was abrogated when catecholamine receptor blockers were added to the bathing solution before EF treatment, suggesting this acute effect, in contrast to the chronic effect, is mediated by catecholamines in EF (Fig. 7, E and F).

EF increases AFC in vivo, but not in vitro by ATII. We investigated the effects of EF using direct measures of fluid transport both in vitro, using ATII, and in vivo, where there are multiple cell types lining the distal lung units. Although we were able to quantitate an apical-to-basolateral fluid transport across ATII monolayers that was ~50% amiloride sensitive, 25% EF did not induce a statistically significant change in fluid transport in vitro with the use of this model system (Fig. 8A). To evaluate the effect of EF in vivo in the adult lung, we instilled EF into the lungs of normal rats. Twenty-four hours later, these rats had significantly increased AFC (Fig. 8B) that was amiloride sensitive (Fig. 8C). This was not seen in control rats receiving comparable instillations of either Ringer-lactate or BSA-Ringer-lactate solutions. Furthermore, rats instilled with heat-treated EF did not have altered AFC relative to control groups. We speculate that the EF-induced increase in AFC is not due to the catecholamines that are present within the EF: the increase in AFC was measured 1 day after instillation of the EF, and catecholamines have a much shorter biological effect on lung function, and also the catecholamine receptor blockade did not alter the effect chronic exposure of ATII to EF. When plasma was instilled into the lungs of normal adult rats, it too increased AFC.

Fig. 5. Biological activity of EF fractionated via Cibacron blue dye affinity chromatography and of commercial rat albumin and globulin preparations. A: relative to the medium control (M), the EF fraction eluted from Affi-Gel blue with 1.4 M NaCl stimulated AIC in rat fetal distal lung epithelial (FDLE) to the same degree as EF (*P < 0.001 compared with medium control, n = 6). Proteins from the flow-through fraction (0.02 M NaCl) and from earlier fractions eluted with 0.25 and 0.5 M NaCl failed to stimulate AIC. Albumin was the most abundant protein in the active fraction (SDS-PAGE; data not shown). B: commercial preparations of rat albumin (9 mg/ml) were tested for biological activity. EF and 96% pure rat albumin (Sigma A-6272) stimulated AIC (*P < 0.001, n = 15); no AIC stimulation was obtained when FDLE were exposed to 99% pure rat albumin (Sigma A-6414). C: exposure of FDLE to a commercial preparation of rat globulins (GL; Sigma G-5015) at 3.6 mg/ml significantly stimulated AIC (*P < 0.01, n = 11).

mainly composed of α-globulins, significantly stimulated AIC relative to medium controls (Fig. 5C).

Globulin increases AIC while decreasing ASC, having no effect on total Iₒ. ATII were treated for ~20 h with increasing concentrations of commercial globulin from 0 to 14.4 mg/ml to investigate the effects of globulins on ion transport. Globulin increased AIC up to the maximum dose of 14.4 mg/ml (4.6 ± 0.5 μA/cm², n = 7, vs. 1.2 ± 0.1 μA/cm² in medium, n = 11, *P < 0.001; Fig. 6). ASC showed a trend of decreasing with increasing globulin dose; however, this effect did not reach significance at the maximum dose (3.5 ± 1.2 μA/cm², n = 7, vs. 5.4 ± 0.6 μA/cm² in medium, n = 11). No significant changes to total current were observed in globulin-treated cells.

EF affects Na⁺-permeant ion channels in the apical membranes of ATII. When primary monolayer cultures of ATII cells that had been grown at an air-liquid interface were
Fig. 7. EF increases the number and open probability of Na\(^+\) channels in ATII cells. A: an 18-h basolateral exposure to 50% EF increased the ATII cells’ apical membrane density of highly Na\(^+\)-selective channels (*\(P < 0.01, n = 14–20\) without changing the number of nonselective cation channels (NSC). The major cation channel was highly selective for Na\(^+\) over K\(^+\) with a Na\(^+\)-to-K\(^+\) permeability ratio (\(P_{\text{Na}}/P_{\text{K}}\)) > 40 and had a unit conductance of 6 pS with mean open and closed times of 1.23 ± 0.418 and 3.64 ± 1.23 s, respectively. The NSC had a \(P_{\text{Na}}/P_{\text{K}}\) close to 1 and a unit conductance of 21 pS with mean open and closed times of 19 ± 6 and 397 ± 172 ms, respectively. B: EF relative to control medium did not change the open probability (\(P_o\)) of the highly Na\(^+\)-selective channel but did decrease the \(P_o\) of the NSC (*\(P < 0.05, n = 9–17\)). C: acute exposure to EF activates ENaC in ATII cells. After control epithelial Na\(^+\) channel (ENaC) activity was measured (untreated), EF was added until it made up 50% of fluid bathing the cell. Cells were exposed to EF apically and basolaterally. A typical recording in which there was a transient decrease in channel activity was followed by an increase to a level greater than before treatment. D: in patches obtained from 6 different lung slices, EF increased channel \(P_o\), significantly (\(P = 0.022\) by Student’s paired \(t\)-test). In the 6 experiments shown, \(P_o\) was calculated from a 2-min period just before addition of EF and then from a 2-min period beginning 10 min after addition. E: activation of ENaC in ATII cells by acute exposure to EF is blocked by catecholamine blockers. ENaC activity was measured in cells treated with 10 \(\mu\)M propanolol, SCH-23390, and sulpiride (after control) and then again after EF was added until it made up 50% of fluid bathing the cell. F: in patches obtained from 8 different lung slices exposed to catecholamine blockers, EF treatment had no effect. In the 8 experiments shown, \(P_o\) was calculated from a 2-min period just before addition of EF and then from a 2-min period beginning 10 min after addition.

**DISCUSSION**

Pulmonary edema, a common disorder occurring throughout life, is associated with significant morbidity and mortality. Since the rate of AFC has been shown to correlate with survival, it is important to know whether our previous studies demonstrating that EF increases ion transport in the perinatal lung (32) were applicable to the postnatal and adult lung. Our present study demonstrates that this is the case. Using different measures of ion transport, we have shown in primary cultures of ATII, in situ lung slices, and in vivo AFC measurements that EF treatment increases lung Na\(^+\) and fluid transport.

We have studied primary cultures of ATII grown under submersion culture or conditions. It is important to recognize that extrapolation to the in vivo situation must be done cautiously and with the recognition that many factors might result in different in vivo and in vitro results. ATII primary cultures contain one cell type, whereas in vivo there are numerous other cell types in the distal lung unit, including ATI, ATII, and Clara cells, which have all been shown to actively transport Na\(^+\) in an apical-to-basolateral direction (36, 37). The contribution of different cells in the distal units or alveolar epithelium is not well defined, although recent in situ lung slice studies suggest that ATII cells quantitatively play an important role in the intact adult lung (18, 19). Patch-clamp studies in ATI have identified HSC, NSC, pimozide-sensitive cation channels, K\(^+\) channels, and the cystic fibrosis transmembrane regulator (CFTR) (18, 19). In addition, cells within their natural environment in vivo are known to have cell-cell interactions and cell-matrix interactions and are exposed to all of the various physiologic circulating hormones and mediators that together may alter their ion-transporting phenotype.

Overall, EF effects on the bioelectric properties of primary cultures of ATII were qualitatively similar to what our group previously observed in FDLE (15, 32). Plasma did not significantly alter AIC, ASC, or total current in ATII, indicating that active factors in EF are inactive or not present in plasma. Commercial rat serum globulins increased AIC but had no effect on total \(I_{sc}\). It may be that factors in EF that are able to induce AIC are also present in the globulin preparation, but at least some factors involved in the modulation of ASC are not.

What component or components of the EF is or are responsible for the induction of ion transport by the perinatal and adult lung epithelia? Heat treatment of EF inactivated the ability of EF to increase AIC in ATII monolayers, as well as in vivo AFC, suggesting that the active factors either are proteins or require proteins to function. This is similar to our in vitro findings using FDLE. Catecholamines do mediate the immediate ATII response to brief exposure to EF in vitro (Fig. 7, E...
Fig. 8. EF does not increase apical-to-basolateral fluid transport in vitro but increases in vivo alveolar fluid clearance (AFC). A: fluid transport was measured using monolayers of ATII cultured on permeable membranes and treated apically and basolaterally with 25% EF. Amiloride (Amil; 100 μM) significantly decreased apical-to-basolateral fluid transport in medium-treated and EF-treated cells; however, EF exposure did not significantly increase fluid transport. *P < 0.05 relative to absence of amiloride for control. §P < 0.05 relative to absence of amiloride for EF-exposed cells. B: in vivo exposure of lung air spaces in rats to EF (n = 15) increased AFC 24 h later relative to Ringer lactate (R; n = 16, P < 0.01) or bovine serum albumin (BSA; n = 5, *P < 0.05). PL, plasma; HT, heat-treated EF. C: AFC was significantly reduced when amiloride was included in the instilled solutions (*P < 0.05). The component of AFC that was amiloride insensitive was not different between the 2 groups.

and F). These results are consistent with in vivo studies showing that catecholamines increase fluid clearance after a short-term exposure (e.g., Refs. 1, 2, 8, 14). In contrast to these short-term studies, our in vivo experiments evaluated the AFC in lungs that had been exposed to EF 24 h before the measurements were carried out. Since any biological effects of the catecholamines would have dissipated by that time, we believe it is the EF, rather than the catecholamines within the EF, that accounted for the increased AFC. In support of this speculation, we have also shown that catecholamines do not explain the EF-induced increase in AIC that was observed in ATII. Specifically, ATII that were exposed to catecholamines for 20 h at levels comparable to those in EF did not show altered Na⁺ transport relative to control groups. Furthermore, addition of catecholamine receptor blockers to the EF did not prevent the majority of the increase in AIC due to EF in ATII monolayers. Studies from others also support this line of argument: when ATII are continuously exposed to terbutaline for 48 h, they do have increased Na⁺/K⁺-ATPase activity; however, 24 h is insufficient time to see this effect (24). In addition, there is evidence that β-adrenergic agonist-stimulated Na⁺ transport is amiloride sensitive (31, 34, 42) and not amiloride insensitive as we have observed in our present studies.

It is more likely that the active component(s) are protein(s) or a protein complex, since our laboratory (32) has previously demonstrated that exposure of the EF to either heat or trypsin abrogates its biological effects. Our present work supports these findings, since fractionation of CHF-induced pulmonary EF demonstrated that the globulin fraction of edema fluid could increase AIC in ATII (Fig. 6). Commercial preparations of rat globulin prepared from rat plasma were found to similarly increase AIC. Rat plasma obtained from the rats with cardiogenic pulmonary edema, which also contains catecholamines, did not significantly alter ion transport in ATII. Since EF is a plasma transudate, these findings suggest that some component of plasma is activated during its movement through the lungs’ interstitium, within the air space, or together with some additional interstitial or alveolar component, is capable of inducing such a response. Support for this is found in work by others who showed that haptoglobin, a common plasma protein, has a modified structure when it is isolated from pulmonary edema fluid (7).

Our present experiments revealed a surprising dose-response effect of EF on the amiloride-sensitive ion transport by ATII. Although at high or low doses of EF, ASC is unchanged, treatment of ATII with moderate doses of EF results in increased ASC (Fig. 1). This type of relationship has been previously described for other compounds. For example, the response of the muscarinic receptor to alkyltrimethylammonium agonists has been shown to vary with different derivatives, and the decyl derivative produces a dose response similar to that we have observed (35). Also, this type of a dose response is seen when increasing amounts of cRNA encoding channel-activating proteases (CAP) are injected into amphibian oocytes (39). Although we have no evidence, it is possible that the effects of EF on ASC we have observed are actually due to two separate pathways, one resulting in increased ASC, for example, plateauing at 25% EF, and the other resulting in decreased ASC throughout the entire EF dose-response curve. This may also explain why globulin treatment inhibited ASC if the ASC inducing pathway was not activated by globulin.

In apparent contrast to our present study, others have recently reported in an abstract that pulmonary EF from acute lung injury (ALI) patients causes cultured human type II cells to downregulate the expression of ENaC and Na⁺/K⁺-ATPase. These studies would suggest that soluble factors in ALI edema fluid may inhibit AFC (20). However, these studies use EF from ALI patients, which contained inflammatory cells and their products, such as cytokines or other mediators, which could lead to cellular damage. This situation is markedly different from our present study, where we used cell-free supernatant EF that had been collected from rats a mere 30 s after the induction of acute CHF. Indeed, in agreement with the ALI studies (20), our laboratory has previously shown that...
more prolonged exposure to products from activated macrophages markedly downregulate amiloride-sensitive Na\(^{+}\) transport (10) and ENaC mRNA levels (11).

Both EF and globulins induced AIC in ATII. Presently, the ion-transporting pathways that are responsible for amiloride-insensitive ion transport and mediators of these pathways are poorly understood. Although in vivo and in vitro experiments have demonstrated the presence of amiloride-insensitive Na\(^{+}\) transport via the Na\(^{+}\)-glucose cotransporter (5, 6), its quantitative contribution to total lung transepithelial ion transport is small (27, 30). For example, it has been shown that addition of glucose to the lung liquid of fetal lambs only induced very small changes to net Na\(^{+}\) flux (3), and our previous work (32) has demonstrated that the Na\(^{+}\)-glucose symporter inhibitor phloretin had no effect on the EF induction of AIC in FDLE. Na\(^{+}\)/H\(^{+}\) exchangers are present in ATII, but they are sensitive to the dose of amiloride that we utilized. In addition, previous experiments in ATII (33) have demonstrated that they do not contribute to net transepithelial Na\(^{+}\) transport. Two studies have reported that dopamine-induced lung fluid clearance is amiloride insensitive (4, 9), but a more recent study suggests a role of dopamine in ENaC regulation (18). The CNGC Na\(^{+}\)-permeant ion channel has been reported to be expressed within the lung and to be relatively insensitive to amiloride (12). CNGC is not active in the rat lung under resting conditions, although a cGMP analog-stimulated increase in lung liquid clearance is blocked by the CNGC inhibitor diltiazem (25). However, our previous studies in FDLE have revealed that diltiazem only blocked a small portion of EF-induced AIC (32), making it unlikely that increased AIC was due to increased CNGC expression. In addition, recent studies suggest that ATII cells contain ENaC and CFTR but few pimozide-sensitive cation channels, which are predominantly present in ATI (19). This further suggests that CNGCs are likely not involved to a significant degree in EF-induced AIC in ATII.

Because of limitations of the various methods used in this study, we were unable to keep our experimental conditions absolutely consistent among different model systems. As a result, there are some differences in the conditions used for different experiments. Our short-circuit current and patch-clamp studies on ATII monolayers were done over similar time periods (18–20 h). However, in situ lung slices could not be exposed to EF overnight because the tissue only remains viable for a few hours when prepared as described. Similarly, for fluid transport measurements, a 20-h period was not sufficient to allow us to quantitate fluid transported, since we were measuring very small changes in fluid volume. Despite these differences among experimental protocols, we have been able to show that EF increased at least one parameter of Na\(^{+}\) transport in all of our studies, with the only exception being the in vitro fluid transport experiments.

Although there was a trend, we were unable to show any effects of EF treatment on fluid transport across ATII cell monolayers in culture. It is possible that this is due to the longer incubation period (48 h) used in these experiments. Our previous work (32) measuring the effect of EF on FDLE monolayers treated for up to 24 h indicates the EF increases AIC in a time-dependent manner. We have not done short-circuit current measurements after a 48-h treatment; however, in fluid transport experiments on FDLE, our laboratory has shown a significant increase in fluid transport after a 48-h period (15). It therefore seems unlikely that the longer time period used for fluid transport experiments in ATII is the reason we did not see a response. It is more likely that we were not able to show an effect in these experiments because of the variability of the assay, where we were attempting to measure very small changes in fluid volume relative to the modest increase in Na\(^{+}\) transport in ATII in response to EF. In contrast, using this method, we have demonstrated an increase in fluid transport across FDLE in response to EF (15). The difference may be due to the more striking effect of EF on Isc in FDLE compared with ATII. However, in vivo studies on healthy adult rats did demonstrate that EF instilled into the lung was sufficient to significantly increase AFC rates relative to control groups (Fig. 8).

In conclusion, using primary cell culture, in situ lung slices, and in vivo experiments, we have shown that CHF-induced pulmonary EF increases distal lung Na\(^{+}\) and fluid transport in the adult lung. We speculate that exposure of normal lung epithelium to EF may initiate a feedback mechanism that augments AFC and that these studies point toward a new therapeutic approach for patients with CHF- or ARDS-induced pulmonary edema and newborn respiratory distress syndrome.

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