Alveolar Epithelial Fluid Clearance Is Mediated by Endogenous Catecholamines at Birth in Guinea Pigs

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Abstract

Transition from placental to pulmonary oxygenation at birth depends on a rapid removal of fetal lung fluid from the developing alveoli. Alveolar fluid clearance was examined in ventilated, anesthetized developing guinea pigs of the ages newborn, 2-d-old, 5-d-old, 30-d-old, and 60-d-old (adult). An isosmolar 5% albumin solution was instilled into the lungs of the guinea pigs; the guinea pigs were then studied for 1 h. Alveolar fluid clearance was measured from the increase in alveolar protein concentration as water was reabsorbed. Newborn guinea pigs had a very high alveolar fluid clearance rate that declined rapidly within the first 5 postnatal days towards adult levels. The high alveolar fluid clearance at birth was apparently mediated by the β-adrenergic system as demonstrated by the elevated plasma epinephrine levels and the increased sensitivity to inhibition by the β-adrenergic antagonist propranolol immediately after birth. Surprisingly, exogenous addition of epinephrine was not able to stimulate alveolar fluid clearance in the newborn lung, but exogenous epinephrine stimulation increased over time to adult levels. The elevated alveolar fluid clearance at birth was associated with a significantly greater amiloride sensitivity in the newborn guinea pig lung. Northern blot analysis of distal lung tissue as well as isolated alveolar epithelial type II cells showed and confirmed higher levels of the α-subunit of the epithelial sodium channel mRNA in the newborn lung that rapidly tapered off toward adult levels. In conclusion, these data demonstrate the importance of the β-adrenergic system and amiloride-sensitive sodium transporting pathways for clearance of fetal lung fluid at birth. (*J. Clin. Invest.* 1998. 101:972–981.) Key words: alveolar epithelium • development • epinephrine • epithelial sodium channel • sodium transport

Introduction

Survival of the newborn is critically dependent on its ability to absorb fetal fluid from the developing air spaces of the lungs at the switch from placental to pulmonary gas exchange. Factors affecting absorption of fluid in newborn lungs are largely unknown. Walters and Olver (1) speculated early that high levels of endogenous catecholamines present at birth may be important for turning on fluid absorption in the developing air spaces of the lungs. Later studies demonstrated that epinephrine was important for clearance of fetal lung fluid at birth (2), though it may not be the only important factor (3). Other maturational hormones, such as glucocorticosteroids and thyroid hormones, may also be important factors regulating lung fluid balance around birth (4). Therefore, there must be a cellular mechanism that can respond to β-adrenergic stimulation in order to increase water reabsorption from the alveoli. Fluid secretion into the air spaces in utero is dependent on chloride secretion (5), and as long as this chloride secretion exists, air spaces will continue to be fluid-filled. As water follows an osmotic gradient generated by ion movements in order for absorption to occur, it is necessary that chloride secretion decreases or that an opposing ion transport system is turned on to generate an inward osmotic gradient. This occurs around birth as the alveolar epithelium switches from chloride secretion to sodium absorption, thus changing from fluid secretion to fluid absorption (5). Very little is known about the extent and efficiency of this sodium transport at birth and immediately thereafter. However, it is known that active sodium transport drives alveolar fluid across the normal healthy adult alveolar epithelium into the lung interstitium and vasculature (6–9). Amiloride-sensitive sodium channels (ENaC)[1] localized to alveolar epithelial type II cells are proposed to be the molecular mechanism involved in apical sodium uptake (9). Three homologous subunits (α, β, and γ-ENaC) comprising such a channel have been cloned recently (10, 11), and their presence has been demonstrated in the lung (12–14). A Na^+,K^+-ATPase on the basolateral side of type II epithelial cells pumps sodium actively into the interstitium (15). Water then moves passively in response to movement of sodium.

Our first objective in these studies was to study alveolar fluid clearance at different developmental stages. Age groups studied were newborn (∼ 4 h from delivery), 2-d-old, 5-d-old, 30-d-old, and adult (60-d-old). We used guinea pigs as our model because their lung development is similar to human lung (16) and also because newborn guinea pigs are large enough to be used in these functional studies. Our goal was to determine the functional mechanism and efficiency of alveolar fluid clearance at birth and the first postnatal week, and up to adulthood. As it has been proposed that catecholamines may be important for regulating alveolar fluid clearance at birth (1), our second aim was to investigate functionally the importance of endogenous β-adrenergic stimulation on the clearance of fetal lung fluid at birth and up to adulthood. Therefore,

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1. *Abbreviations used in this paper:* db, dibutyryl; ENaC, epithelial sodium channel; RITC, rhodamine B isothiocyanate.

we used the β-adrenergic antagonist propranolol to investigate whether endogenous catecholamines could be responsible for clearing the air spaces from fluid at birth. We also took plasma samples from each developmental stage for measurements of endogenous epinephrine levels. In adult lungs, it has been demonstrated that epithelial sodium transport via amiloride-sensitive sodium channels provides much of the driving force for alveolar water reabsorption (9). Therefore, our third aim was to use amiloride and investigate whether the amiloride-sensitive fraction of alveolar fluid clearance varied with development and thereby indicated alterations in function and/or expression of the amiloride-sensitive alveolar epithelial sodium–transporting pathways. Also, Northern blot analysis was carried out to investigate changes in the mRNA levels of the α subunit of the cloned amiloride-sensitive ENaC (α-ENaC) of developing guinea pigs.

Methods

Animals

Guinea pigs of the Dunkin-Hartley strain ($n = 129$, Sahlins Försöksdjursfarm AB, Malmö, Sweden) were used in the study. Both male and female guinea pigs were used. The Ethical Review Committee on Animal Experiments at Lund University had approved the experiments.

Surgery

The animals were anesthetized by intraperitoneal injections of pentobarbital sodium (30 mg/kg body wt; Apoteksbolaget, Umeå, Sweden). An endotracheal tube (PE-200/PE-240; Clay Adams, Becton Dickinson, Parsippany, NJ) was inserted into the trachea through a tracheostomy. Carotid artery catheterization (PE-40/PE-50 catheter; Clay Adams, Becton Dickinson) was done for monitoring hemodynamic parameters (heart rate and systemic blood pressure), blood sampling, and drug administration. Pancuronium bromide (Pavulon®, 0.3 mg/kg body wt/h; Organon-Teknika, Boxtel, The Netherlands) was given through the arterial catheter for neuromuscular blockade. The animals were connected to a constant-volume ventilator (Harvard Apparatus, Inc., South Natick, MA) with an inspired O_2 fraction of 1.0 and with tidal volumes adjusted to register peak pressures of 10 cm H_2O during the baseline period. Hemodynamic parameters and airway pressures were measured using calibrated pressure transducers (UFI model 1050BP; BioPac Systems, Inc., Goleta, CA) connected to analog-to-digital converters and amplifiers (models MP100 and DA100, respectively; BioPac Systems, Inc.) and recorded continuously on an IBM computer with Acknowledge software (version 3.2; BioPac Systems, Inc.). The animals were placed on a slanting board in the left decubitus position and covered with a heating pad to maintain normal body temperature.

General experimental protocol

All animals had at least a 20-min baseline period for stabilization of hemodynamic parameters. 10 min into the baseline period, rhodamine B isothiocyanate (RITC)-conjugated dextran 70,000 (2.5 mg/ml in 0.9% NaCl; Sigma Chemical Co., St. Louis, MO) was injected through the arterial catheter, and after another 10 min, a blood sample was taken for fluorescence measurements. Fluid instillation was then done as follows. The animals were disconnected briefly from the ventilator, and the 5% albumin instillate (see below for details about preparation of instillates) was instilled into the lungs over 10–15 s using a soft instillation catheter placed immediately above the bronchial carina. Animals < 30 d old were given 10 ml/kg body wt of fluid as instillate, while 30-d-old and adult (60-d-old) guinea pigs were given 6 ml/kg body wt. It has been demonstrated that volume of instilled fluid does not affect alveolar fluid clearance (6, 17). After fluid instillation, the animals were reconnected immediately to the ventilator and remained ventilated for the 1-h experiment. At the end of the experiment, a blood sample was taken, and the animals were given an overdose of pentobarbital sodium intraarterially. The lungs and heart were removed en bloc from the thoracic cavity through a midline sternotomy. A sample of alveolar fluid was aspirated by gently advancing a sampling catheter into a wedged position in a distal airway in the lungs. Protein concentrations in instillates and air space samples as well as in final plasma samples were measured by the Lowry method (18) modified for microtiter plates. Plasma epinephrine levels were measured in selected baseline blood samples from all age groups with an HPLC technique using fluorescence detection (19, 20). Both lungs were then homogenized separately for fluorescence measurements and wet-to-dry weight measurements.

Preparation of instillates

The 5% albumin instillate solution was prepared by dissolving 50 mg/ml BSA (Sigma Chemical Co.) in an aqueous solution of 0.9% NaCl. For the studies of dependence of alveolar fluid clearance on endogenous epinephrine, we added the general β-adrenergic antagonist, propranolol (Sigma Chemical Co.), at the 10^{-4} M concentration to the 5% albumin instillate solution. For the studies of whether exogenous epinephrine addition could stimulate alveolar fluid clearance, we added epinephrine (NM Pharma, Stockholm, Sweden) at the 10^{-6} M concentration to the 5% albumin instillate solution. In some studies, we added 10^{-4} M dibutyryl(db)-cAMP (Sigma Chemical Co.) to the instilled fluid. For determination of fractional inhibition of amiloride on alveolar fluid clearance, we added amiloride (Sigma Chemical Co.) at the 10^{-3} M concentration to the 5% albumin instillate solution. We used amiloride at the 10^{-3} M concentration because ~ 50% of amiloride is protein bound, and another significant fraction escapes from the air spaces, resulting in functional concentrations closer to 10^{-4} M (21, 22).

Specific experimental protocols

The following experimental groups and specific experimental protocols were used in the study.

Group 1. Control studies (newborn: n = 6; 2-d-old: n = 6; 5-d-old: n = 6; 30-d-old: n = 4; 60-d-old (adult): n = 7). After the baseline period, the guinea pigs were instilled with 6–10 ml/kg body wt of the 5% albumin instillate in the lungs. Newborn, 2-d-old, and 5-d-old guinea pigs were instilled with 10 ml/kg body wt in order to be able to recover any alveolar fluid at the end of the study, as preliminary experiments indicated a very high alveolar fluid clearance in the younger animals. 30-d-old and adult guinea pigs were instilled with 6 ml/kg body wt. Furthermore, earlier studies have demonstrated that surface area is of little if any importance for determining the rate of alveolar fluid clearance in several animal species (6, 15, 17). The guinea pigs were studied for 1 h and then exsanguinated and processed as described in the general experimental protocol above. Some guinea pigs ($n = 3$ in each group for distal lung preparations; $n = 3$ in each group for alveolar epithelial type II cell isolation) from each age group were also used for the isolation and analysis of α-ENaC mRNA expression during postnatal development.

Group 2. Endogenous epinephrine studies (newborn: n = 5; 2-d-old: n = 4; 5-d-old: n = 4; 30-d-old: n = 4; 60-d-old: n = 4). After the baseline period, the guinea pigs were instilled as above with 6–10 ml/kg body wt of the 5% albumin instillate containing 10^{-4} M propranolol into the lungs. The guinea pigs were studied for 1 h and then exsanguinated and processed as described in the general experimental protocol above. Also, in three guinea pigs from each age group, endogenous epinephrine and norepinephrine levels were measured in plasma.

Group 3. Exogenous epinephrine studies (newborn: n = 4; 2-d-old: n = 4; 5-d-old: n = 4; 30-d-old: n = 4; 60-d-old: n = 4). After the baseline period, the guinea pigs were instilled with 6 ml/kg body wt of the 5% albumin instillate containing 10^{-6} M epinephrine into the lungs. The guinea pigs were studied for 1 h and then exsanguinated and processed as described in the general experimental protocol above.

Group 4. db-cAMP studies under β-adrenergic blockade (newborn: n = 4; adult: n = 4). Inhibition of phosphodiesterase activity has been shown to be essential to maintain db-cAMP effectiveness (23). Therefore, 15 min into the baseline period, the animals were given a bolus injection of 10^{-4} M aminophylline to inhibit phosphodiesterases. After the baseline period, the guinea pigs were instilled with 6–10 ml/kg body wt of the 5% albumin instillate containing 10^{-4} M propranolol, 10^{-4} M db-cAMP, and 10^{-4} M aminophylline into the lungs. Aminophylline (10^{-4} M) was then also infused continuously throughout the 1 h experimental time. The guinea pigs were studied for 1 h and then exsanguinated and processed as described in the general experimental protocol above.

Group 5. Amiloride studies (newborn: n = 4; 2-d-old: n = 4; 5-d-old: n = 4; 30-d-old: n = 4; 60-d-old: n = 4). After the baseline period, the guinea pigs were instilled with 6–10 ml/kg body wt of the 5% albumin instillate containing 10^{-3} M amiloride into the lungs. The guinea pigs were studied for 1 h and then exsanguinated and processed as described in the general experimental protocol above.

Alveolar fluid clearance calculation

Clearance of fluid from the distal air spaces of the lungs was measured by the increase in alveolar protein concentration of the instilled solution over 1 h. The increase in protein concentration due to removal of water from the air spaces is a direct reflection of alveolar fluid clearance, as has been demonstrated previously (6, 24, 25). Data is presented as a ratio of final to instilled protein concentration or as alveolar fluid clearance in percent instilled volume. This method is accurate for measuring alveolar fluid clearance, since protein leakage due to epithelial and endothelial permeability dysfunction was negligible (see Results). However, the term alveolar fluid clearance does not imply that all fluid is reabsorbed at the alveolar level. Some fluid may be reabsorbed by the distal airways. Ballard and colleagues (26) have demonstrated that the distal airways can transport sodium and thereby potentially absorb luminal fluid. Alveolar fluid clearance (*AFC*) is calculated from the following equation:

$$AFC = [(V_I - V_F)/V_I] \times 100\,(\%\text{ of instilled volume}),$$

where V_I is instilled volume (in milliliters), and V_F is final alveolar volume (in milliliters) calculated from the protein concentrations in instilled and final alveolar fluids. Because the lung is fluid-filled in utero (5), we had to take into account that a certain fraction of this fluid could be still present in the lungs at birth. This fluid is virtually free of protein and will not add protein to the albumin concentration (measured as total protein concentration) in the instillate. In contrast, it will dilute the protein concentration in instillates and thereby influence the calculations of alveolar fluid clearance differently depending on volume of fluid present in the different age groups. To control for this, we therefore had separate groups of animals at each developmental stage that were instilled with the 5% albumin solution; the fluid was then withdrawn immediately. This was done as follows. After anesthesia and tracheostomy, the lungs were instilled with 1.5–2 ml of the 5% albumin instillate, and this fluid was aspirated and reintroduced four times before the final sample was taken. The whole procedure took ~ 1–2 min, and during this time it is unlikely that significant amounts of protein left or entered the air spaces or that significant volumes of fluid were reabsorbed from or secreted into the air spaces. As protein would not cross the alveolar epithelial barrier during this short time, any change in protein concentration would represent a dilution by preexisting fluid. Knowing this, we could calculate the preexisting fluid volume by the following relationship:

$$V_L = (V_I \times C_I)/C_{L,}$$

where V_L is preexisting lung fluid volume (in milliliters), V_I is instilled fluid volume (in milliliters), C_I is protein concentration in instilled fluid (in milligrams per milliliter), and C_L is protein concentration in aspirated fluid (in milligrams per milliliter). The preexisting fluid volume was used to correct the instilled protein concentrations by the dilution of the instillate that would occur if there were fluid already in the lung before instillation of the 5% albumin solution.

Lung vascular leakage

Clearance of the vascular tracer RITC-dextran 70,000 from the blood circulation into the lung extravascular spaces was measured as extravascular plasma equivalents. Total lung extravascular RITC-dextran accumulation was calculated by taking the total lung RITC-dextran and subtracting vascular space RITC-dextran. RITC-dextran was measured fluorometrically by a spectrophotofluorometer (CytoFluor 2300; Millipore Corp., Bedford, MA). Vascular space RITC-dextran was calculated by multiplying the mean RITC-dextran in plasma by the calculated lung plasma volume, as has been done previously (27, 28). Extravascular lung accumulation of RITC-dextran was expressed as plasma equivalents, i.e., milliliters of plasma that account for lung fluorescence. Since dextran and albumin have similar molecular masses (70 vs. 68 kD), movement of dextran across endothelial–epithelial barriers was considered equal to movement of albumin into lung extravascular spaces.

Lung tissue and alveolar epithelial type II cell preparation

In whole lung studies, fetuses and neonates were killed, and the lungs were excised. Distal lung tissue was prepared by removing the lung lobes and teasing the tissue from the second to third generation bronchi. In addition to epithelial cells, this preparation also includes blood cells and cells derived from the endothelium, mesenchyme, airway, and blood capillaries. Alveolar epithelial type II cells were isolated from fetal guinea pigs by a method described previously by Monaghan and colleagues (29) with a few modifications for neonatal samples. Neonates were anesthetized with a 1-ml intraperitoneal injection of 25% Hypnorm, 25% Hypnovel (F. Hoffmann-La Roche, Welwyn Garden City, UK), 50% H_2O heparinized mixture. Tracheal cannulas were used for the instillation of 5–10 ml ice-cold solution (5 mM KCl, 140 mM NaCl, 10 mM Hepes, 20 mM glucose, and 2 mM EDTA) into the neonatal lungs. The solution was left for 10 min, removed, and followed with an additional five washes of similar volumes to remove alveolar macrophages. Cells were then differentially digested from their underlying elastin-rich matrix by incubation with elastase (Elastin Products Co., Inc., Pacific, MO) as described for fetal tissue (29), and the resultant high purity alveolar epithelial type II cell pellet (30) was resuspended in TRIzol RNA extraction buffer (Sigma Chemical Co.).

Preparation and analysis of RNA

Lung tissue and alveolar epithelial type II cell RNA was prepared by the acid guanidinium thiocyanate-phenol-chloroform method (31) using TRIzol reagent (Sigma Chemical Co.). Total RNA was resuspended in RNase-free H_2O and quantified by analysis of optical density at 260 nm by ultraviolet (UV) spectrophotometry.

RNA samples were analyzed by Northern or slot blots using standard protocols. Briefly, 5 μg lung tissue or 30 μg alveolar epithelial type II cell total RNA was denatured with 50% formamide, 6.5% formaldehyde in Mops buffer (0.1 M Mops, pH 7.0, 40 mM sodium acetate, 5 mM EDTA, pH 8.0). The samples were loaded onto formaldehyde gels (1.1% agarose, 33% formaldehyde, in Mops buffer, pH 7.0) and electrophoresed in Mops buffer for 3–4 h. Gels were then washed for 20 min in RNase-free water followed by a 20-min submersion in 0.1 N NaOH, followed by 45 min in 20× SSC. The RNA was capillary-blotted onto Hybond nylon membrane (Amersham International, Little Chalfont, UK) with 20× SSC overnight.

Slot blots were performed by an adaptation of the method of Kacimi and colleagues (32) using 5 μg RNA per slot, blotted onto Hybond-N blotting membrane (Amersham International) and using a blotting apparatus (Millipore Corp.). Filters from both protocols were removed, rinsed in 2× SSC, and the RNA was cross-linked to the membrane by UV irradiation (UVP Cross-Linker; Hoeffer Scientific Instruments, San Francisco, CA).

A guinea pig–specific α-ENaC probe of 761 bp was prepared by reverse transcription PCR from guinea pig lung RNA using primers designed from rat sequences. The sense primer corresponds to bases 1,059–1,079, and the antisense corresponds to bases 1,800–1,820 of α-ENaC (GenBank accession no. X70497). The sequence of this probe was 85% homologous to the rat sequence for this region and hybridizes to an mRNA of 3.7 kb in guinea pig lung (our unpublished observations). An 18s ribosomal RNA oligonucleotide probe (kindly provided by Dr. H. McArdle, The Rowett Institute, Aberdeen, UK) was used to correct for any loading variance between the RNA samples.

Probes were labeled with ^{32}P-dCTP using a Multiprime labeling kit (Amersham International). The 18s deoxyoligonucleotide terminal phosphate was replaced with [γ-^{32}P]ATP using a polynucleotide kinase (Promega UK, Southampton, UK) catalyzed reaction. Unincorporated nucleotides were removed by applying the labeled mixes to a microspin Probe-Quant column (Pharmacia Biotech Ltd., St. Albans, UK). High specific activity probes (10^8 cpm/μg DNA) were used for the hybridization protocols. Filters were prehybridized with rapid hybridization buffer (Amersham International) for 15–30 min at 65°C. Hybridization was carried out in the same buffer for 3 h at 65°C with denatured double-stranded α-ENaC or the 18s oligonucleotide probe using 5×10^6 cpm/ml hybridization buffer. Blots were washed sequentially at 65°C in 2× SSC plus 0.1% SDS for 20 min, and in 1× SSC plus 0.1% SDS and 0.1× SSC plus 0.1% SDS for 15 min each. The blots were then wrapped in plastic film and analyzed with an imager (Hewlett-Packard Co., Andover, MA) for 1–4 h or exposed to autoradiographic film at −70°C for 1–5 d.

Quantification of the RNA was carried out either by image analysis of ^{32}P disintegrations in the specific area of the hybridized product using the imager or by densitometry analysis of scanned film-positive autoradiographs using public domain NIH image software.

Statistics

All data are presented as means±SD. The data were analyzed with one-way ANOVA and Tukey's test post hoc. Two groups were compared with unpaired Student's *t* test. Differences were considered significant when a *P* value of < 0.05 was reached.

Results

Alveolar fluid volume in developing lungs. Since lungs are fluid-filled in utero, the presence of preexisting fluid in the lungs had to be taken into consideration in our experiments. We accounted for this by studying the dilution of the instilled 5% albumin solution after immediate retrieval; since preexisting lung fluid would be protein-free (5), any dilution would represent a volume of preexisting alveolar fluid. We found, as expected, that newborn lungs contained the highest volume of preexisting lung fluid (1.11±0.14 ml/kg body wt in the newborn lungs and 0.50±0.09 ml/kg body wt at 2 d), and that this volume decreased rapidly to become insignificant at postnatal day 5. This represented a 15% dilution of the instilled fluid's protein concentration in the newborn lung, a dilution that decreased rapidly to only a 4% dilution in 2-d-old guinea pigs. We used this volume to correct the alveolar fluid clearance calculations for an initial dilution for the existence of fluid in the alveoli already at the start of the experiments.

Alveolar fluid clearance during early postnatal development. Animals in all age groups were instilled with the 5% albumin solution. A sample from the distal air spaces was collected by aspiration of the remaining alveolar fluid after 1 h. Results are presented as the ratio between final to instilled protein concentrations and as alveolar fluid clearance in percentage of instilled volume. The final to instilled protein concentration was highest (2.54±0.31) in newborn guinea pigs, showing that 62±6% of instilled volume was removed from the air spaces within 1 h (Fig. 1). The clearance tapered rapidly down to the level of alveolar fluid clearance in adult guinea pigs (38±5%) within the first postnatal 5 d. Measurements of endogenous levels of circulating catecholamines at each developmental stage demonstrated that the circulating levels of catecholamines, i.e., epinephrine, in the newborn guinea pigs were high (Fig. 2, *inset*). Furthermore, as alveolar fluid clearance tapered down towards adult levels, circulating epinephrine levels decreased towards adult levels also. No changes with development were observed in circulating norepinephrine levels.

Sensitivity to endogenous β-adrenergic stimulation during development. Since endogenous epinephrine plasma levels in the newborn were high, and alveolar fluid clearance in the adult guinea pigs could be stimulated by exogenous administration of epinephrine (33), we studied whether alveolar fluid

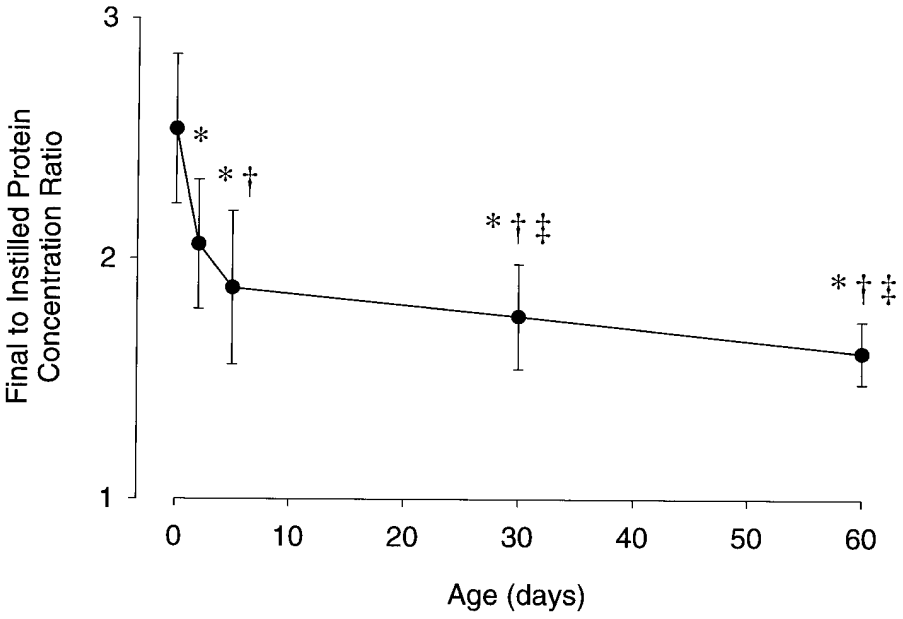

Figure 1. Alveolar epithelial fluid clearance over 1 h in developing guinea pigs after instillation of the 5% albumin solution expressed as final to instilled protein concentration ratio. Clearance of fluid from the distal air spaces was significantly higher in newborn guinea pigs than in older animals. The alveolar fluid clearance tapered rapidly down to adult levels within the first 5 postnatal days. Values are mean±SD (newborn: $n = 6$; 2 d: $n = 6$; 5 d: $n = 6$; 30 d: $n = 4$; 60 d: $n = 7$); $^*P < 0.05$ compared to newborn, $^\dagger P < 0.05$ compared to 2-d-old, $^\ddagger P < 0.05$ compared to 5-d-old lung (ANOVA with Tukey's test post hoc).

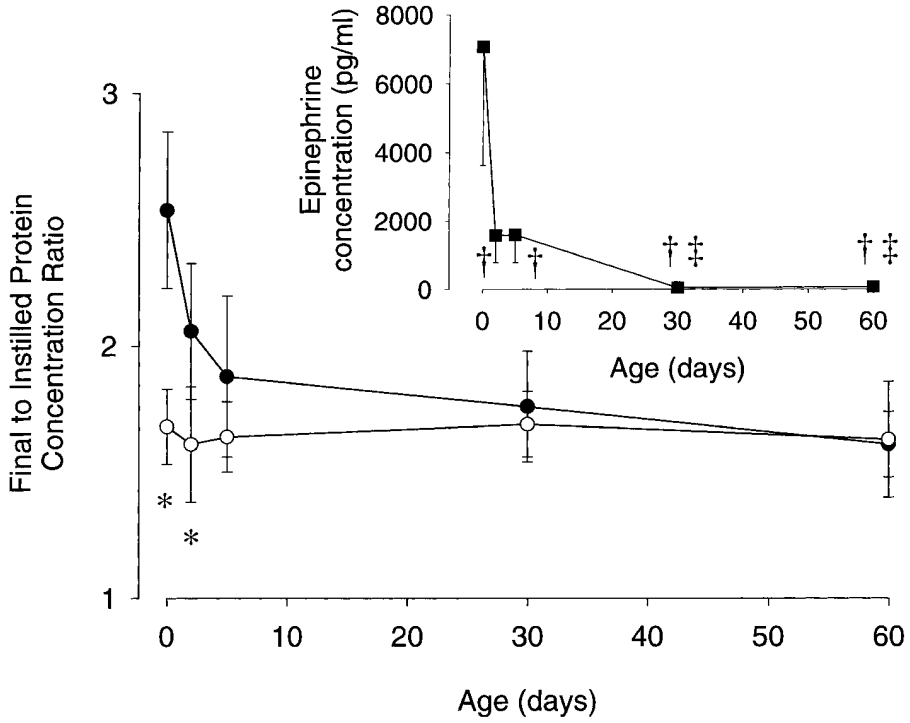

Figure 2. Alveolar epithelial fluid clearance over 1 h in developing guinea pigs after instillation of the 5% albumin solution with propranolol (10^{-4} M) added, expressed as final to instilled protein concentration ratio. Plasma epinephrine levels were also measured in the different age groups (*inset*). Clearance of fluid from the distal air spaces was inhibited by propranolol to a significantly higher degree in newborn guinea pigs than in older animals. The inhibition of alveolar fluid clearance by propranolol tapered rapidly down to adult levels within the first 5 postnatal days concomitantly with a significant decrease in plasma epinephrine levels. In 30-d-old and adult guinea pigs, no inhibition by propranolol on alveolar fluid clearance was seen. Also, epinephrine levels were at baseline levels in those age groups. *Inset*, Plasma epinephrine levels were elevated significantly at birth and then declined rapidly towards baseline levels within the first 5 postnatal days. Values are mean±SD (control groups [●] as in Fig. 1; newborn: $n = 5$; 2 d: $n = 4$; 5 d: $n = 4$; 30 d: $n = 4$; 60 d: $n = 4$); propranolol inhibition (○) $P < 0.05$ compared to control animals (ANOVA with Tukey's test post hoc); epinephrine plasma levels (■) $^{\dagger}P < 0.05$ compared to newborn, $^{\ddagger}P < 0.05$ compared to 2- and 5-d-old guinea pigs (ANOVA with Tukey's test post hoc).

clearance would be sensitive to inhibition by the β-adrenergic antagonist propranolol and if such a sensitivity would vary with development. We also wanted to find out if the high alveolar fluid clearance in the newborn lung was related to endogenous β-adrenergic stimulation from the observed elevated plasma epinephrine levels at birth. Therefore, we instilled developing guinea pigs with the 5% albumin instillate containing 10^{-4} M propranolol, and the animals were studied as above for 1 h. Addition of propranolol to the instilled fluid abolished completely the elevated alveolar fluid clearance in the newborn guinea pigs (Fig. 2). Inhibition by propranolol decreased with increasing age (Fig. 2) and was completely absent in the adult lungs. In fact, the decrease in sensitivity to propranolol inhibition almost paralleled the decrease in endogenous circulating epinephrine levels. As long as epinephrine levels were elevated, propranolol inhibited alveolar fluid clearance (Fig. 2).

Sensitivity to exogenous β-adrenergic stimulation during development. The ability of the developing guinea pig lung to respond with an increased alveolar fluid clearance to exogenously administered epinephrine was also investigated. In this experimental series, guinea pigs from each age group were instilled with the 5% albumin instillate solution containing 10^{-6} M epinephrine and were studied over 1 h. Addition of exogenous epinephrine did not stimulate alveolar epithelial fluid clearance in newborn guinea pigs (Fig. 3). However, in 2- and 5-d-old guinea pigs, exogenous addition of epinephrine resulted in a weak tendency of stimulation (6–7%) of alveolar epithelial fluid clearance (Fig. 3). At 30 d of age, a stimulatory response by epinephrine could be observed (19%), approximately half of that observed in the adult (35%) guinea pig (Fig. 3).

Stimulation of alveolar epithelial fluid clearance by db-cAMP during β-adrenergic receptor blockade. To investigate whether epinephrine stimulates alveolar fluid clearance by increasing intracellular cAMP, we used a cell-permeable form, db-cAMP. In the newborn lung, addition of db-cAMP to the instilled fluid after β-adrenergic blockade by propranolol restored alveolar fluid clearance to > 90% of baseline levels (Table I). In the adult guinea pigs, db-cAMP addition induced a similar response to that demonstrated with exogenous epinephrine (Table I).

Involvement of amiloride-sensitive sodium channels during development. In adult lungs, it has been demonstrated that active sodium transport across the alveolar epithelium is a key mechanism for creating the driving force for alveolar water reabsorption (for a review, see reference 15). Moreover, even though amiloride has been demonstrated to inhibit alveolar fluid absorption in both newborn and adult lungs (21, 22, 25), little is known about possible variations in amiloride sensitivity during postnatal development. Therefore, we used amiloride to determine the contribution of amiloride-sensitive sodium channels for alveolar fluid clearance during early postnatal development. Addition of 10^{-3} M amiloride to the 5% albumin instillate demonstrated that a significantly larger fraction of alveolar fluid clearance in newborn guinea pigs was sensitive to amiloride inhibition than in older lungs (Fig. 4). The sensitivity

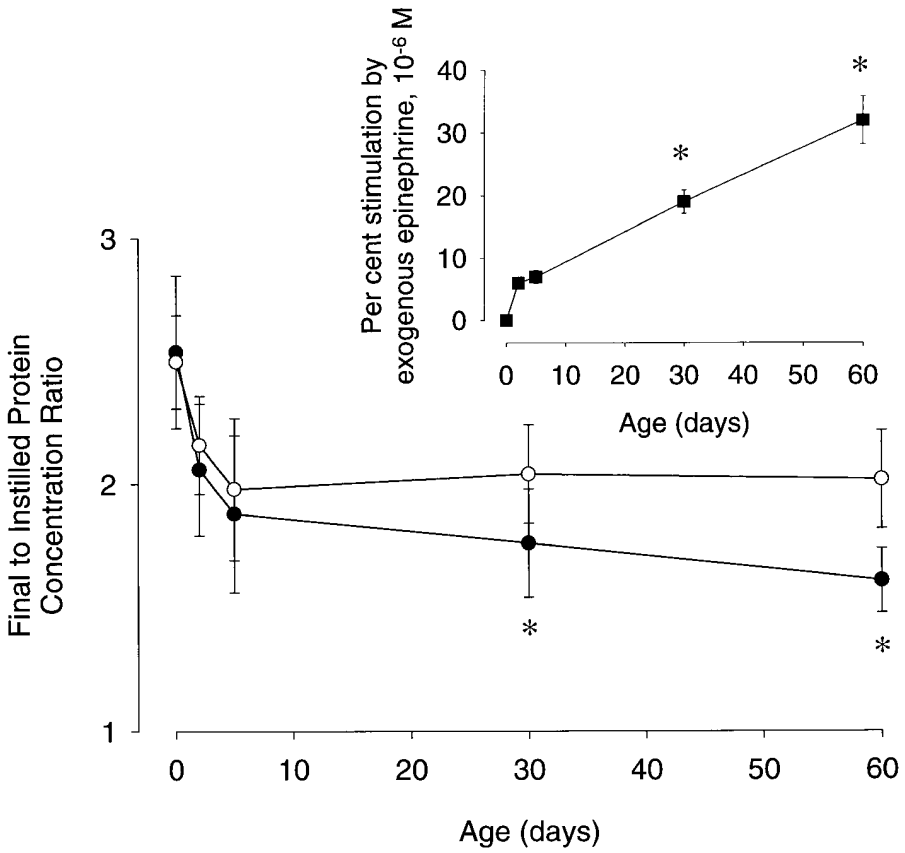

Figure 3. Alveolar epithelial fluid clearance over 1 h in developing guinea pigs after instillation of the 5% albumin solution with 10^{-6} M epinephrine (○), expressed as final to instilled protein concentration ratio. Clearance of fluid from the distal air spaces was stimulated by exogenous epinephrine addition from postnatal day 5 up to adult age. *Inset*, Stimulation of alveolar fluid clearance by exogenous epinephrine in percent stimulation from control animals at each developmental stage. Stimulation was evident only in the 30-d-old and adult guinea pigs. Values are mean±SD (control groups [●] as in Fig. 1; newborn: $n = 4$; 2 d: $n = 4$; 5 d: $n = 4$; 30 d: $n = 4$; 60 d: $n = 4$), $^*P < 0.05$ compared to control guinea pigs (ANOVA with Tukey's test post hoc).

to amiloride inhibition decreased rapidly after birth (Fig. 4, *inset*) and was similar to that in adult guinea pigs already between days 2 and 5.

Expression studies of α-ENaC mRNA by Northern and slot blot. To investigate a possible mechanism responsible for the elevated alveolar epithelial fluid clearance at birth, we used Northern and slot blot analysis to investigate any differences in α-ENaC mRNA expression in distal lung and alveolar epithelial type II cell preparations during early postnatal development. The results showed that in distal lung, levels of α-ENaC mRNA fall from day 1 postnatal, with a decline of 22%, towards adult levels at 7 d postnatal (Fig. 5). This decline was even more pronounced in the alveolar epithelial type II cell preparation, where α-ENaC mRNA falls from term to reach significantly lower ($P < 0.05$) levels by 4–5 d postnatal (Fig. 5). The decline in α-ENaC mRNA expression in both preparations correlates well with the functional data and its relative amiloride sensitivity during development.

Table I. Alveolar Fluid Clearance and db-cAMP

Group	*n*	Final to instilled protein concentration ratio
Newborn	6	2.54±0.31*
Newborn + propranolol	5	1.68±0.15
Newborn + propranolol + db-cAMP	4	2.24±0.27*
Adult	7	1.61±0.13
Adult + propranolol	4	1.63±0.19
Adult + propranolol + db-cAMP	4	1.91±0.12‡

Alveolar fluid clearance after instillation of 5% albumin containing the membrane-permeable cAMP analogue db-cAMP (10^{-4} M) after β-adrenergic blockade with 10^{-4} M propranolol in guinea pigs. Guinea pigs were also instilled with the 5% albumin solution both alone and containing 10^{-4} M propranolol. Alveolar fluid clearance is expressed as the final to instilled protein concentration ratio. $^*P < 0.05$ compared to newborn plus propranolol; $^‡P < 0.05$ compared to adult plus propranolol (ANOVA with Tukey's test post hoc).

Developmental changes in lung endothelial leakage and hemodynamic parameters. We investigated whether developmental changes in lung endothelial leakage existed by using RITC-conjugated dextran 70,000 as a vascular marker molecule. We found that lung endothelial leakage was low and similar in all age groups (0.1–0.2 ml). Therefore, we could neglect this as a possible reason for the developmental differences in alveolar fluid clearance. Furthermore, this low lung endothelial leakage confirms that our fluid instillation into the developing lung did not damage lung endothelial–epithelial barriers.

There were no alterations in measured hemodynamic parameters that could be attributed to the experimental procedures or produce the observed differences in alveolar fluid clearance. The only significant hemodynamic effect observed from our manipulations was a reduction in heart rate in propranolol-treated animals (data not shown). However, it is very unlikely that this would affect net alveolar fluid clearance, since earlier studies have shown that neither blood flow nor ventilation affect alveolar fluid clearance over 4 h in sheep and human lungs (34–36).

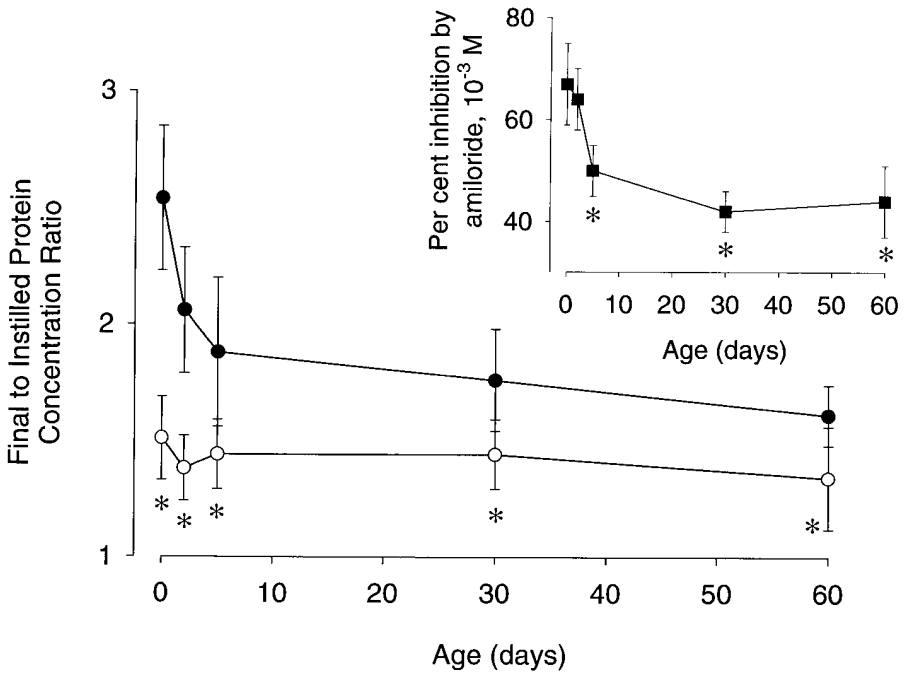

Figure 4. Alveolar epithelial fluid clearance over 1 h in developing guinea pigs after instillation of the 5% albumin solution with the sodium channel inhibitor amiloride (○) added at the concentration 10^{-3} M and expressed as final to instilled protein concentration ratio. Clearance of fluid from the distal air spaces was inhibited to a significantly higher degree by amiloride in newborn guinea pigs than in older animals. The inhibition of alveolar fluid clearance tapered off rapidly to the inhibition seen in the adult animals within the first 5 postnatal days. *Inset*, Inhibition of alveolar fluid clearance by amiloride in percent inhibition from control animals at each developmental stage. Values are mean±SD (control groups [●] as in Fig. 1; newborn: $n = 4$; 2 d: $n = 4$; 5 d: $n = 4$; 30 d: $n = 4$; 60 d: $n = 4$), $^*P < 0.05$ compared to control guinea pigs (ANOVA with Tukey's test post hoc).

Discussion

We found dramatic differences in the ability of the alveolar epithelium to clear excess fluid from the air spaces during early postnatal development in the guinea pig. What is the factor regulating alveolar fluid clearance at birth? In adult guinea pigs, it has been demonstrated that addition of exogenous β-adrenergic agonists can stimulate reabsorption of fluid from the air spaces (33); this has also been shown in other animal species (7, 17, 24, 27, 36, 37). In addition, exogenous epinephrine can stimulate reabsorption of fetal lung fluid near term in other animal species (2, 5, 38, 39). Thus, endogenous cate-

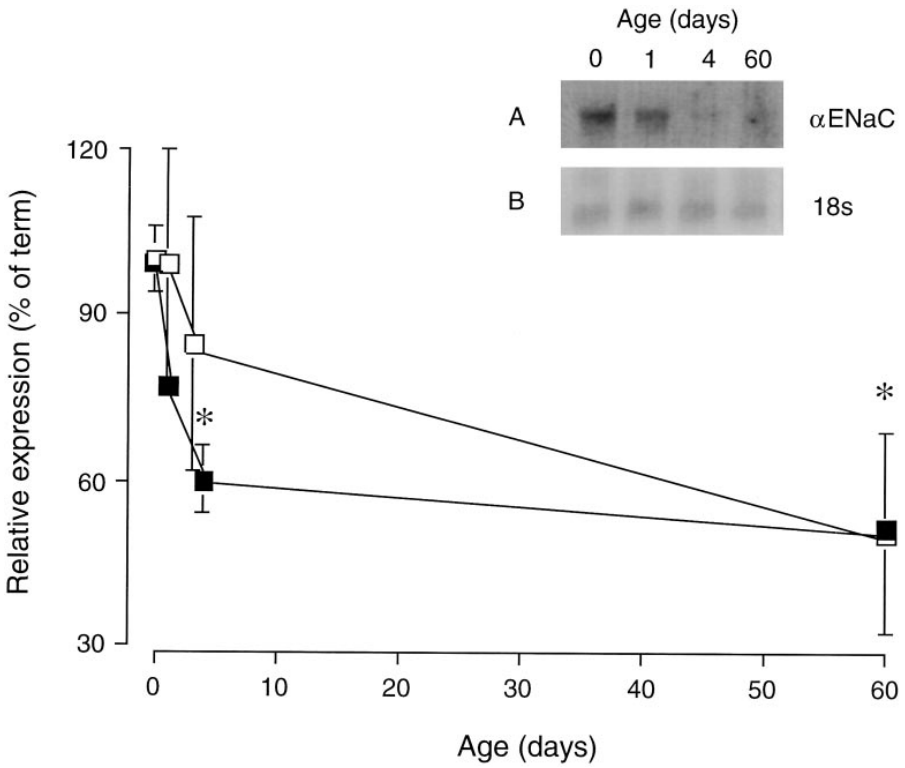

Figure 5. Expression of α-ENaC mRNA in distal lung and alveolar epithelial type II cell preparations from guinea pig lung. Densitometry quantitation or image analysis was carried out on slot blots or Northern blots loaded with 5 or 30 μg total RNA extracted from distal lung (□) or alveolar epithelial type II cell preparations (■) from guinea pigs at different developmental time points. Blots were adjusted for loading by comparison to 18s ribosomal RNA levels. *Inset*, A typical Northern blot of total RNA (30 μg) extracted from alveolar epithelial type II cell preparations at different developmental time points. (*A*) The α-ENaC probe recognized an mRNA of ~3.7 kb that declined postnatally from peak expression levels at term. (*B*) The same samples hybridized with an 18s oligonucleotide probe. α-ENaC mRNA percentage expression is calculated relative to term (day 68) to standardize between experiments for $n = 3$ blots in all cases, except for the alveolar epithelial type II cell preparations from day 1 postnatal and adult guinea pigs where $n = 2$. In distal lung, levels of α-ENaC mRNA fall from 1 d postnatal, with a decline of 22%, towards adult levels at 7 d postnatal. This decline is more dramatic in the alveolar epithelial type II cell preparation, where α-ENaC mRNA falls from term to reach significantly lower levels ($^*P < 0.05$, $n = 3$) by 4–5 d postnatal.

cholamines, whose levels increase in the blood circulation during labor and delivery (2), may play an important role in lung fluid clearance at birth. However, even though some data are present, there is little functional evidence linking endogenous catecholamine levels and absorption rates at this time. Therefore, we investigated whether endogenous epinephrine and norepinephrine could be involved in regulating alveolar fluid clearance at birth by two methods. First, we measured endogenous epinephrine and norepinephrine plasma levels in developing animals and tried to correlate these levels to the ability to clear excess alveolar fluid. Second, we inhibited β-adrenergic receptor stimulation by adding propranolol to the instilled fluid. We found that endogenous epinephrine levels indeed correlated well with an increased ability of the lung to clear excess alveolar fluid. Endogenous epinephrine levels were elevated in newborn animals as alveolar fluid clearance was high (Fig. 2 and Fig. 2, *inset*). Both epinephrine levels and alveolar fluid clearance decreased rapidly between birth and postnatal day 5. As a confirmation of the importance of epinephrine and the β-adrenergic receptor for clearance of fetal lung fluid at birth, fluid absorption in newborn lungs was found to be more sensitive to propranolol inhibition than older lungs (Fig. 2). Propranolol inhibited all of the elevated clearance by the neonatal time points and tapered off to no inhibition in adult lungs. This inhibition decreased concomitantly with epinephrine levels. Thus, propranolol was only able to inhibit alveolar fluid clearance when endogenous epinephrine plasma levels were elevated above the levels in adult guinea pigs. Studies have shown that the number of β-adrenergic receptors in guinea pig lung remains fairly constant during development from newborn to adult animals, and that the small increase in absolute numbers can be explained by increases in alveolar surface area (40). Therefore, our results of a stimulation of alveolar epithelial fluid clearance at birth cannot be explained solely by changes in receptor number. Instead, it is more likely that the stimulation of alveolar epithelial fluid clearance in the newborn is due to increased receptor stimulation by the elevated plasma epinephrine levels at birth.

Surprisingly, the newborn lung did not respond with an increased alveolar epithelial fluid clearance to the exogenous epinephrine addition as the adult guinea pig lung does. This could have several explanations. First, alveolar epithelial fluid clearance was already maximally stimulated from the elevated plasma levels of endogenous epinephrine seen in the newborn guinea pigs and thus could not be further stimulated. Second, there are additional adrenoceptor-mediated pathways in the adult lung that may act differently. However, since propranolol inhibited all the elevated fluid clearance induced by exogenous epinephrine in the adult guinea pig lung (33), and endogenous epinephrine apparently stimulated alveolar epithelial fluid clearance in the newborn lung, the latter explanation is less likely. Therefore, the reason for the lack of a response in the newborn lung from exogenous epinephrine is probably that alveolar epithelial fluid clearance was already maximally stimulated by the elevated endogenous epinephrine plasma levels, and thus alveolar fluid clearance cannot be further stimulated.

In support of this hypothesis, we found that db-cAMP instilled into the lung induced similar levels of alveolar fluid clearance to those found in the newborn lung and after β-adrenergic stimulation in the adult lung (Table I). Taken together, these data suggest that epinephrine stimulation of fluid clearance is mediated via β-adrenoceptor stimulation of intracellular cAMP. The fact that db-cAMP restored alveolar fluid clearance levels but did not induce additional stimulation in the newborn lung indicates that fluid clearance from the air spaces was already maximally stimulated by the endogenous epinephrine. Conversely, negligible levels of endogenous epinephrine in the adult lung result in a decreased receptor stimulation, which facilitates activation of β-adrenoceptor–mediated alveolar fluid clearance by exogenous epinephrine or db-cAMP.

What is the driving force to clear fetal lung fluid at birth? In adult lungs, active sodium transport through amiloride-sensitive sodium channels has been demonstrated as a key mechanism for creating the driving force for alveolar epithelial water reabsorption (9, 15). Also, in newborn guinea pig lungs, amiloride has been demonstrated to impair reabsorption of fetal lung fluid (21), and in fetal lungs, amiloride has been shown to inhibit completely alveolar fluid absorption (39). However, little if any functional data are available on possible differences in sensitivity to amiloride inhibition during postnatal lung development. Therefore, we investigated whether amiloride inhibited different fractions of alveolar fluid clearance at different developmental stages. We instilled developing guinea pigs with the 5% albumin instillate containing 10^{-3} M amiloride and studied them for 1 h. We found that a significantly larger fraction of alveolar fluid clearance was inhibited by amiloride in newborn than in older guinea pigs (Fig. 4). This indicates that amiloride-sensitive pathways in newborn guinea pig lungs were upregulated and thereby provided a greater osmotic force driving water reabsorption into the blood circulation more efficiently. We then wanted to confirm our functional findings with a molecular investigation of the mechanism underlying the amiloride-sensitive fluid absorption. We found that the levels of α-ENaC mRNA were high at birth and then tapered off rapidly thereafter in both distal lung preparations and alveolar epithelial type II cell preparations. These results paralleled our functional data, suggesting that ENaC is at least partly responsible for the rapid fluid clearance at birth in the guinea pig; as the level of expression falls, amiloride sensitivity falls. In summary, an increased amiloride-sensitive sodium transport is responsible for clearing developing guinea pig lungs of fetal lung fluid at birth.

However, there appears to be a difference in expression levels with increasing postnatal age between the whole distal lung preparation and the isolated alveolar epithelial type II cell preparation, though the general downward trend is the same in both preparations. This apparent difference may be caused by the fact that α-ENaC mRNA is expressed in more than one cell type in the lung; for example, α-ENaC mRNA has been shown (14) to be expressed in tracheal, bronchial, and bronchiolar epithelial cells, in which it may be less markedly developmentally regulated, in addition to its expression in the alveolar epithelium. In the distal lung preparation, we cannot exclude a contribution from airway α-ENaC to the developmental expression profile. In contrast, the alveolar epithelial type II cell preparation provides a more accurate profile of what is happening in the alveolar epithelium during early postnatal development.

Amiloride does not seem to inhibit as large a fraction of alveolar fluid clearance in adult rat lungs after stimulation with β-adrenergic agonists or other stimulatory factors (25, 37) as it does in the fetus (39) and in this study of developing newborn

guinea pig lungs. Thus, the increased sensitivity to amiloride inhibition observed at term in this study cannot be solely a result of elevated epinephrine levels, but must also derive from an increase in the sodium channel number in the cell membrane. It seems likely that an increased expression and/or function of amiloride-sensitive ENaCs would be responsible for the elevated capacity of the alveolar epithelium to clear excess alveolar fluid at birth. In a recent study using isolated rat fetal distal lung epithelial cells, it was suggested that the β-adrenergic agonist terbutaline may promote trafficking of amiloride-sensitive sodium-permeable nonselective cation channels to the apical cell membrane (41). Rao and Cott (42) also demonstrated an increase in the short-circuit current of fetal alveolar epithelial cells after stimulation with terbutaline over a 1-h span that was inhibitable by propranolol, although the proportion of amiloride-sensitive blockable transport increased as term approached. In conclusion, although terbutaline and other β-adrenergic agonists will increase channel open probabilities and open times, the increase in amiloride sensitivity in the newborn compared with the adult lung is more likely due to the increased synthesis and insertion of channels into the alveolar epithelial cell membrane. Further support for the latter comes from our molecular studies of α-ENaC mRNA expression, which suggest that an increased expression of α-ENaC at birth is responsible for the high alveolar fluid clearance at that time. The increase in mRNA could lead to the increased protein transcription and translocation of protein to the apical membrane observed earlier after β-adrenergic stimulation (41) and then to activation via the elevated epinephrine levels as suggested previously (42). Whether high circulating levels of epinephrine induced the expression of α-ENaC at birth cannot be deduced from these studies, although other investigators have suggested that the preterm rise in α-ENaC may be the result of other factors (4, 43, 44). Also, it was demonstrated recently that transgenic mice that were made deficient in α-ENaC lacked the ability to clear the lungs of fetal lung fluid at birth, and these mice died shortly after birth with significant pulmonary edema (45). All this evidence suggests a critical role for the amiloride-sensitive ENaC in preparation for pulmonary gas exchange.

In most other animal species studied, intraalveolar amiloride inhibits alveolar liquid clearance to a similar extent to that seen in the adult animals in this study. Thus, alveolar liquid clearance is driven by stimulation of both amiloride-sensitive and amiloride-insensitive pathways. One possible explanation for the fractional inhibition by amiloride may be the existence of as yet not identified cation channels that are insensitive to amiloride. Recent data (46, 47) have identified the existence of a rod-type cyclic nucleotide–gated cation channel in the alveolar epithelium that could potentially play a role in lung fluid clearance.

In conclusion, we found functional and molecular evidence for stimulated alveolar fluid clearance in newborn guinea pig lungs that rapidly decreased towards adult levels within days after birth. This elevated alveolar fluid clearance depended on elevations in plasma levels of epinephrine in newborn guinea pigs. Both alveolar fluid clearance and circulating epinephrine levels decreased dramatically between postnatal days 1 and 5. Surprisingly, exogenous addition of epinephrine did not stimulate alveolar epithelial fluid clearance in the newborn lung, probably because alveolar fluid clearance was already maximally stimulated by the elevated endogenous epinephrine levels in the plasma. The increased ability to clear fluid from the distal air spaces of the lungs was demonstrated to correlate with an increased expression of alveolar amiloride-sensitive ENaCs in the newborn lung. The sensitivity to amiloride inhibition and α-ENaC expression decreased dramatically between postnatal days 2 and 5.

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