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Intrapulmonary Terbutaline and Aminophylline Decrease Lung Liquid in Fetal Lambs

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ABSTRACT. To see if phosphodiesterase inhibition might enhance the effect of β-adrenergic stimulation on fetal lung liquid secretion, we studied the independent and combined effects of intrapulmonary terbutaline and aminophylline on net production of lung luminal liquid over time (Jv) in fetal lambs with chronically placed tracheal loop catheters. We calculated Jv during baseline and experimental periods (90–120 min each) by measuring serial concentrations of ^{125}I-albumin, an impermeant tracer that was well mixed in the luminal liquid. In 21 experiments, tracheal instillation of terbutaline (10^{-5} M) decreased Jv from 11 ± 1 (mean ± SEM) to −3 ± 2 mL/h. In six other studies, aminophylline (10^{-3} M) alone had no significant effect on Jv. In 12 experiments, we gave the two drugs sequentially: terbutaline decreased Jv from 11 ± 2 to −3 ± 2 mL/h and aminophylline further decreased Jv to −8 ± 2 mL/h. Amiloride (10^{-4} M), an inhibitor of epithelial Na^+ transport, reversed the combined effect of terbutaline and aminophylline, increasing Jv to 8 ± 1 mL/h. Thus, phosphodiesterase inhibition enhances the β-adrenergic effect of terbutaline on Na^+-dependent absorption of liquid from the lung lumen of fetal lambs. (*Pediatr Res* 29: 357–361, 1991)

Abbreviations

Jv, net production of lung luminal liquid over time

Potential air spaces of the fetal lung are filled with liquid that must be removed at birth to allow the switch from placental to pulmonary gas exchange. In fetal sheep, i.v. infusion of epinephrine or isoproterenol either inhibits secretion of liquid into the lung lumen or causes its absorption, an effect that propranolol prevents (1, 2). This β-adrenergic effect on lung liquid movement can be blocked by amiloride, an inhibitor of epithelial Na^+ transport (3). In addition, dibutyryl-cAMP, when instilled into the lung lumen at a 10^{-4} M concentration, either reduces net production of lung liquid or causes absorption (4, 5).

The physiologic effects of β-adrenergic agonists are linked to intracellular production of cAMP by adenylate cyclase (6). Pulmonary β-adrenergic receptors increase in concentration near the end of gestation in fetal sheep (7), and stimulation of these receptors increases production of cAMP in fetal lung (8, 9). The purpose of this work was to see if phosphodiesterase inhibition, by preventing the breakdown of cAMP, might enhance the effect of β-adrenergic stimulation on Na^+-dependent absorption of fetal lung liquid. We therefore studied the independent and combined effects of a β-adrenergic agonist (terbutaline) and a phosphodiesterase inhibitor (aminophylline) on net production of lung luminal liquid in fetal lambs. Terbutaline decreased the rate of formation of lung liquid, aminophylline alone caused no significant change, and the two drugs together had a synergistic effect, consistently causing absorption of liquid from the lung lumen. Amiloride blocked the action of terbutaline and aminophylline, causing resumption of liquid secretion.

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MATERIALS AND METHODS

Animal preparation. We surgically prepared fetal lambs at an average gestational age of 127 d (range 122–133 d, term = 147 d). We chose this gestational age to facilitate surgery and allow sufficient time for recovery from surgery and studies to be performed before delivery. Suffolk and mixed breed sheep were mated on a specific day to permit precise assessment of gestational age. On the day of surgery, the ewes received 500 mg of ketamine intramuscularly, followed by either spinal anesthesia with 1% tetracaine or general anesthesia with 1% halothane and nitrous oxide delivered with air and supplemental oxygen through a piston-type mechanical ventilator. We opened the uterus through a midline abdominal incision, injected 1% lidocaine s.c. into a fetal hindlimb (omitted when general anesthesia was used), and threaded polyvinyl catheters through an artery and vein into the fetal aorta and vena cava. Through a second uterine incision, we inserted into the fetal trachea a saline-filled silicone rubber catheter (Silastic medical grade tubing; Dow Corning, Midland, MI; outer diameter 5.0 mm, inner diameter 2.6 mm, vol 17 mL) that formed a loop between the distal and proximal portions of the trachea. The ends of the catheter were situated just above the tracheal bifurcation and just below the vocal cords, allowing normal outflow of lung liquid. We placed a smaller polyvinyl catheter (outer diameter 2.3 mm, inner diameter 1.3 mm) in the lumen of the distal trachea for subsequent pressure monitoring. All fetuses had a catheter sutured to the outside of the chest, with the tip of the catheter in the amniotic sac, providing a zero reference for subsequent pressure measurements. Skin incisions were closed and all catheters were tunneled through the uterine and abdominal walls, which were doubly oversewn to prevent fluid leakage.

We injected penicillin and kanamycin into the amniotic sac (1 000 000 U of penicillin and 400 mg of kanamycin) and fetal vein (300 000 U of penicillin and 30 mg of kanamycin) at the time of surgery and daily thereafter. The ewes also received 5 mL of a mixture of penicillin and dihydrostreptomycin (Combiotic, 200 000 U/mL of procaine penicillin G and 250 mg/mL of dihydrostreptomycin sulfate, Pfizer, New York, NY) and 600 mg of kanamycin intramuscularly each day. Vascular catheters

were flushed with isotonic saline and filled with heparin daily. Operative procedures and experimental protocols were approved by the Committee on Animal Use at the University of California San Francisco.

Experimental design. Animals had at least 3 d to recover from surgery before experiments began. The ewes were kept in a mobile cage with access to food and water, and their fetuses were healthy, as assessed by normal arterial blood gas and pH measurements. During all studies, we measured vascular, amniotic, and tracheal pressures through fluid-filled catheters connected to calibrated pressure transducers (BT3DC; Statham Instruments, Oxnard, CA) and an eight-channel amplifier-recorder (model 7D; Grass Instruments, Quincy, MA). We obtained samples of arterial blood hourly for measurement of pH, arterial O_2 and CO_2 tensions, hematocrit, and plasma protein concentration.

To begin each study, we interrupted the tracheal loop catheter at its midpoint and attached to the caudal end a three-way stopcock connected to a 60- or 35-mL plastic syringe. We clamped the cephalad portion of the tracheal catheter and covered it with sterile gauze for the remainder of the experiment. At the completion of each experiment, the two ends of the tracheal catheter were united with a sterile plastic connector.

At the start of each experiment, we obtained samples of fetal and maternal plasma and fetal lung liquid for measurement of background radioactivity. We then withdrew 30–60 mL of lung liquid, to which we added 1–2 μCi of ^{125}I-labeled human serum albumin (Mallinckrodt Inc., St. Louis, MO) for reinfusion into the trachea. We thoroughly mixed the ^{125}I-albumin in the lung liquid by repetitive withdrawal and reinfusion over a 10- to 20-min period. Thereafter, we repeated this mixing procedure twice during each 10-min period, and we removed 0.5- to 2-mL samples of liquid every 10 min for measurement of radioactivity in duplicate 100-μL aliquots (gamma counter, Beckman Instruments, Palo Alto, CA). We also obtained samples of fetal blood for measurement of plasma radioactivity, which did not exceed background counts during the time course of these experiments. In two experiments, we collected lung lymph, as previously described (10), and observed no increase in radioactivity of lymph over the time course of the study. The size of lung liquid samples was chosen to keep the actual volume of luminal liquid relatively constant throughout the experiments. We usually achieved this by taking 1–2 mL every 10 min; sample size was reduced to 0.5 mL every 10 min during periods of net liquid absorption.

We measured Jv, as described below, first during a 90- to 120-min control period, and then during one or more experimental periods of similar duration. Drugs were injected into the luminal liquid and mixed thoroughly by repetitive withdrawal and reinfusion of the liquid during the first 10 min of each experimental period.

We did a total of 51 experiments on 26 animals. In six control experiments, we monitored Jv for 6 h without injecting any drugs. We determined the effect on Jv of terbutaline alone in 21 studies and the effect of aminophylline alone in six studies. In 12 other studies, we examined the effect on Jv of aminophylline given 90–120 min after terbutaline. To see if the terbutaline effect on Jv was constant for at least as long as the time period after addition of aminophylline, we did three control experiments in which we determined the effect on Jv of terbutaline for 180 min. In 10 of the combined drug experiments, we tested the effect of amiloride in the presence of terbutaline and aminophylline. In six other experiments, we studied the effect of propranolol, given before terbutaline in three studies and after terbutaline in three studies. We performed multiple experiments on six of the 26 sheep. When multiple studies were performed on a single animal, we allowed at least 1 full day to elapse between experiments. We allowed 2–3 d between experiments involving terbutaline alone and 6 d between combined drug experiments that were repeated on the same sheep. When multiple studies were performed on a single animal, we withdrew and discarded as much of the lung liquid as possible at the end of each study to hasten removal of residual drugs.

We instilled the drugs through the tracheal loop directly into the lung. Drug doses were selected to achieve predetermined concentrations derived from the estimated volume of lung liquid. Approximate initial drug concentrations were as follows: terbutaline, 10^{-5} M; aminophylline, 10^{-3} M; amiloride, 10^{-4} M; and propranolol 5×10^{-5} M. Preliminary dose-response experiments showed that the maximal effect of terbutaline was achieved at a concentration between 10^{-5} and 10^{-4} M. The dose-response relationship for terbutaline was constant at concentrations ranging from 0.3×10^{-5} M to 2×10^{-5} M. The usual dose of terbutaline needed to achieve a concentration of 10^{-5} M was about 0.7 mg. The amount of aminophylline required to achieve a concentration of 10^{-3} M was about 40 mg. The dose-response relationship for aminophylline did not change over a 10-fold range of concentrations. Higher doses of aminophylline were not used to avoid potential toxicity. The amiloride dose was chosen to achieve an initial lung liquid concentration similar to that used by Olver *et al.* (3) to block epinephrine-induced absorption of lung liquid in fetal sheep. The propranolol dose, 1–2 mg, was sufficient to cause a sustained decrease in heart rate without producing hypotension or acidosis.

In 11 of the 12 combined drug experiments, we used ion-selective electrodes (Stat profile-5 analyzer, Nova Biomedical, Waltham, MA or Na/K/Cl stat analyzer, model 644, Ciba-Corning Diagnostics, Medfield, MA) to measure the concentrations of Na^+, K^+, and Cl^- in samples of plasma and lung liquid. We measured hematocrit by spinning samples in a microcentrifuge (IEC, Needham Heights, MA), and we used a refractometer (American Optical, Keene, NH) to measure plasma concentrations of total protein.

Data analysis. We calculated the volume of liquid within the lung lumen at the start of each experiment by determining the concentration of ^{125}I-albumin after thorough mixing. We then calculated the cumulative volume of lung liquid at each time point based on the dilution of ^{125}I-albumin, with appropriate corrections for the radioactive counts and liquid volumes removed with previous samples. We then calculated by least squares regression analysis the rate of change in cumulative lung liquid volume over time. Jv represents the sum of liquid secretion and absorption, processes that may coexist within the lung. A positive value for Jv indicates net production and a negative value represents net absorption of liquid.

Statistics. Data presented in the text, tables, and figures are mean values ± 1 SEM. We used a paired t test to compare net liquid production rates before and after a single drug was given. When multiple drugs were tested sequentially in a single experiment, we used a Bonferoni correction for multiple comparisons (11). We accepted a p value of <0.05 as indicative of statistical significance for single comparisons, with appropriate corrections for multiple comparisons.

RESULTS

Control experiments. In six control experiments during which we measured Jv for 6 h without giving any drugs, there was no detectable change in Jv between the first 3 h (17 mL/h) and last 3 h (17 mL/h) of study. The average gestational age of the fetuses used for these experiments was 130 d (range: 128–134 d).

Terbutaline experiments. Terbutaline caused an abrupt decrease of Jv in each of 21 experiments. In 11 studies (Fig. 1), net secretion (positive slope) switched to net absorption (negative slope) after terbutaline. In 10 other experiments, terbutaline reduced net liquid production without causing absorption. On average, terbutaline decreased Jv from 11 ± 1 to −3 ± 2 mL/h (Table 1). This response to terbutaline appeared stable over 180 min (Jv for 0–90 min, −6 ± 7 mL/h; Jv for 90–180 min, −7 ± 6 mL/h; $n = 3$).

The gestational age of the terbutaline-treated fetuses averaged

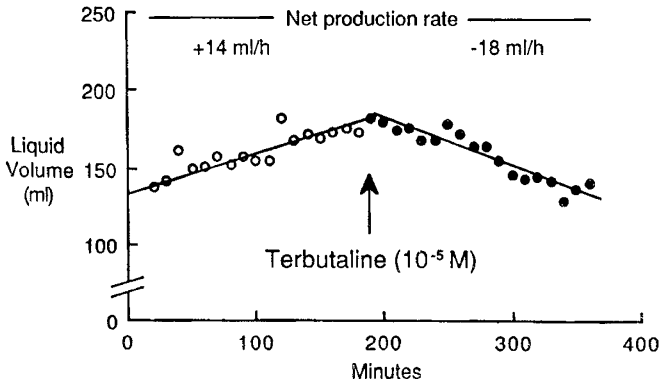

Fig. 1. Effect of terbutaline (10^{-5} M) on Jv in one of 21 experiments. Plotted measurements of liquid volume are cumulative and include the sample volumes removed before and including each time point. Jv for baseline (○) and terbutaline (●) periods were 14 mL/h (secretion) and −18 mL/h (absorption), respectively.

Table 1. *Jv in fetal lambs**

Experimental period	Number of experiments	Jv (mL/h)
Baseline	21	11 ± 1 †
Terbutaline	21	−3 ± 2 †
Baseline	6	17 ± 2
Aminophylline	6	18 ± 4
Baseline	12	11 ± 1 †
Terbutaline	12	−3 ± 3 †
+Aminophylline	12	−8 ± 2 †
+Amiloride	10	8 ± 1 †

* Data are mean values ± 1 SEM.

† Significant difference between successive experimental periods, $p < 0.05$.

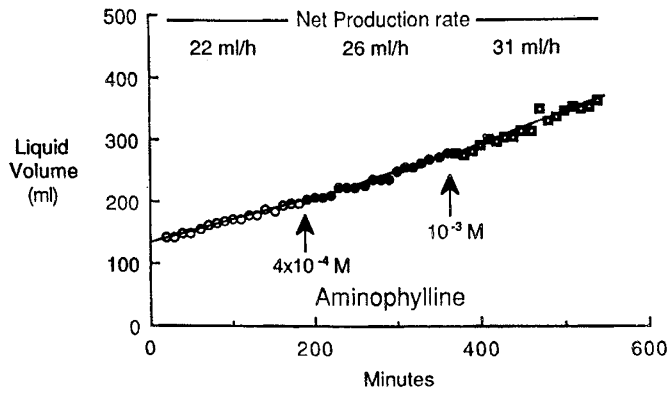

Fig. 2. Effect of aminophylline (at two concentrations) on Jv in one of six experiments. Plotted measurements of liquid volume are cumulative and include the sample volumes removed before and including each time point. Jv for the baseline period (○) was 22 mL/h, low-dose aminophylline period (●) 26 mL/h, and higher dose aminophylline period (□) 31 mL/h.

132 d (range: 126–137 d). There was a statistically significant positive correlation between gestational age and the magnitude of the terbutaline-induced reduction in Jv ($r = 0.5$). The average dose of terbutaline (0.7 ± 0.1 mg) produced an initial lung liquid concentration of 1.0 ± 0.5 × 10^{-5} M. Terbutaline caused a small but statistically significant increase in heart rate (baseline 172 ± 4 beats/min *versus* terbutaline 180 ± 4 beats/min), but did not affect blood pressure, pH or blood gas tensions.

Aminophylline experiments. Aminophylline had no significant effect on Jv (Fig. 2, Table 1). The average gestational age of these six fetuses was 133 d (range: 128–136). Aminophylline alone had no significant effect on heart rate or blood pressure, but it was associated with small decreases in arterial O_2 tension (baseline 2.4 ± 0.3 kPa *versus* aminophylline 2.1 ± 0.3 kPa) and arterial CO_2 tension (baseline 6.4 ± 0.1 kPa *versus* aminophylline 6.1 ± 0.3 kPa).

Combined drug experiments. In these 12 studies, terbutaline decreased Jv from 11 ± 1 to −3 ± 3 mL/h, and addition of aminophylline further reduced Jv to −8 ± 2 mL/h (Table 1, Fig. 3). The average gestational age of these fetuses was 132 d (range: 126–137 d), and there was no significant correlation between gestational age and the magnitude of the effect of terbutaline-aminophylline on Jv.

In 10 of these experiments, addition of amiloride switched the flow of lung liquid from net absorption to net secretion (Jv 8 ± 1 mL/h). In five related studies, we examined the effect of amiloride alone on Jv and found no significant change compared with baseline. In the combined drug experiments, the initial drug concentrations were 1.0 ± 0.1 × 10^{-5} M terbutaline, 1.6 ± 0.7 × 10^{-3} M aminophylline, and 1.2 ± 0.1 × 10^{-4} M amiloride. Neither terbutaline nor aminophylline nor amiloride caused statistically significant changes in blood pressure, heart rate, or blood gas tensions.

Propranolol-terbutaline experiments. In six studies (131 ± 1 d gestation), propranolol (4.0 ± 0.4 × 10^{-5} M) inhibited the effect of terbutaline (0.8 ± 0.2 × 10^{-5} M) on Jv. In three studies, propranolol alone had no significant effect on Jv (baseline 6 ± 5 mL/h, postpropranolol 5 ± 4 mL/h); subsequent addition of terbutaline did not change Jv (postterbutaline 5 ± 2 mL/h). For the three studies in which propranolol was given after terbutaline, Jv was 9 ± 2 mL/h during the baseline period, 1 ± 5 mL/h after terbutaline, and 7 ± 1 mL/h after propranolol. Propranolol significantly slowed fetal heart rate (172 ± 5 to 148 ± 5 beats/min) without changing either vascular pressures, pH, or blood gas tensions.

Electrolyte measurements. We measured concentrations of Na^+, K^+, and Cl^- in samples of lung liquid and plasma obtained from 11 of the 12 combined drug experiments. Baseline plasma electrolyte concentrations were Na^+ 144 ± 5 mmol/L, K^+ 4.1 ± 0.6 mmol/L, and Cl^- 105 ± 3 mmol/L. Baseline lung liquid electrolyte concentrations were Na^+ 149 ± 1 mmol/L, K^+ 5.5 ± 0.3 mmol/L, and Cl^- 156 ± 2 mmol/L. There were no significant changes in any of the lung liquid ion concentrations after terbutaline alone (Na^+ 149 ± 1 mmol/L, K^+ 5.3 ± 0.3 mmol/L, and Cl^- 158 ± 2 mmol/L). Lung liquid Na^+ concentration decreased significantly after adding aminophylline (146 ± 1 mmol/L) and increased after adding amiloride (148 ± 1 mmol/L). Lung liquid K^+ concentration decreased significantly after

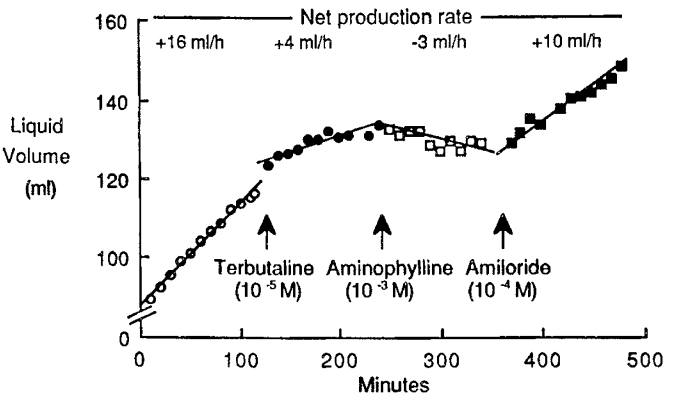

Fig. 3. Effect of terbutaline and aminophylline on Jv in one experiment. Plotted measurements of liquid volume are cumulative and include sample volumes removed before and including each time point. Jv for the baseline period (○) was 16 mL/h, terbutaline period (●) 4 mL/h, terbutaline-aminophylline period (□) −3 mL/h (absorption), and postamiloride period (■) 10 mL/h (secretion).

adding amiloride (5.7 ± 0.3 mmol/L to 5.1 ± 0.3 mmol/L). In all of these studies, the concentrations of Cl^- and K^+ were greater in lung liquid than they were in plasma, as previously reported (12, 13).

DISCUSSION

The results of this work show that terbutaline and aminophylline together stimulate Na^+-dependent absorption of lung liquid in late-gestation fetal lambs. This synergism supports the hypothesis that β-adrenergic agonists act through increased production of cAMP to induce removal of liquid from the lung lumen. The stability of the response to terbutaline over 180 min suggests that the change in Jv after adding aminophylline was not due to a delayed effect of terbutaline. The absence of detectable radioactivity in blood and the maintenance of ion concentration gradients across the epithelium suggest that this effect was not caused by disruption of the epithelial barrier. Amiloride inhibition of the combined effects of terbutaline and aminophylline also indicates that stimulated absorption of lung liquid is the result of transepithelial Na^+ movement in the direction of the interstitium.

The effect on net production of lung liquid by intrapulmonary terbutaline is similar to the effect previously observed with i.v. epinephrine (1, 4, 14). The fact that β-adrenergic blockade with propranolol inhibited the action of terbutaline implies that this effect is mediated specifically through β-adrenergic receptors in the lung. Moreover, the observation that propranolol was inhibitory when given either before or after terbutaline suggests that the β-adrenergic response did not cause irreversible changes in epithelial cell function.

We delivered both terbutaline and aminophylline directly into the lungs to concentrate their effects on the respiratory epithelium and to minimize hemodynamic perturbations. Berthiaume *et al.* (15) used a similar experimental approach to study the effects of β-adrenergic stimulation on lung liquid clearance in adult sheep. These investigators instilled endogenous plasma into one lobe of the lung and found that intrapulmonary terbutaline hastened liquid clearance despite a progressive rise in protein concentration within the luminal liquid. Amiloride (10^{-4} M) inhibited this terbutaline-induced acceleration of liquid clearance.

We found that the effect of terbutaline on lung liquid production was gestation-dependent, confirming previous observations that i.v. epinephrine and dibutyryl-cAMP have a greater effect in causing lung liquid absorption toward term than they do earlier in gestation (1, 4, 5, 14). Previous studies of the ontogeny of β-adrenergic receptors in the lungs of several species, including sheep, indicate that the concentration of β-adrenergic receptors in the lung increases with advancing gestation (7, 9, 16–18). In fetal sheep, the concentration of pulmonary β-adrenergic receptors doubles between 125 and 135 d gestation (7), which might account for the increased effect of terbutaline that we observed over this range of gestation.

Aminophylline had no significant effect on production of lung liquid in our lambs. In studies with cultured rat epithelial cells, two other phosphodiesterase inhibitors, theophylline (10^{-6} M) and isobutylmethylxanthine (5×10^{-4} M), caused significant increases in dome formation within cell monolayers, consistent with stimulated movement of Na^+ and water across the basal surface of the cells (19). These changes were associated with increased concentrations of intracellular cAMP. The apparent difference between these results and our *in vivo* observations is unexplained, but it may be related to a low basal production rate of cAMP in the fetal sheep lung, such that phosphodiesterase inhibition had little effect. It is also possible that fetal and adult lung epithelium respond differently to cAMP or phosphodiesterase inhibition, or that there are species-related differences in pulmonary epithelial cell responsiveness to aminophylline. An alternative explanation is that leakage of aminophylline from the fetal lung lumen may have reduced the effective concentration of the drug at the epithelial surface. This explanation is unlikely, however, inasmuch as no change in liquid production occurred over a 10-fold concentration range. In addition, the estimated concentration of aminophylline was 1000 times greater than the concentration of theophylline needed to increase intracellular cAMP in cultured alveolar cells (19).

The effect of amiloride in blocking liquid absorption that was stimulated by terbutaline and aminophylline implies a Na^+-dependent process. Our findings, however, do not clarify the specific pathways by which epithelial Na^+ uptake occurred because the concentration of amiloride that we used (10^{-4} M) was sufficient to block both apical Na^+ channels and Na^+-H^+ exchange (20). Amiloride alone caused no significant change in baseline Jv. This finding confirms previous observations by Olver *et al.* (3) and indicates that apical Na^+ uptake is not an important force in the regulation of liquid movement across the unstimulated fetal lung epithelium at this gestational age. As in previous studies by other investigators (3–5), amiloride blocked nearly all of the liquid absorption associated with β-adrenergic stimulation. The small but consistent decrease in Na^+ concentration after addition of aminophylline in the presence of terbutaline and the rise in Na^+ concentration after addition of amiloride support the idea that liquid absorption was related to Na^+ transport. The importance of the decrease in K^+ concentration after amiloride is unclear, but it might reflect a reduction in lung epithelial cell Na^+-K^+-ATPase activity, with diminished cell uptake and release of K^+. These results confirm the importance of Na^+-dependent pathways in lung liquid clearance associated with conditions that cause increased production of cAMP.

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