Phosphatidylinositol 4,5-bisphosphate stimulates alveolar epithelial fluid clearance in male and female adult rats

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Kooijman EE, Kuzenko SR, Gong D, Best MD, Folkesson HG. Phosphatidylinositol 4,5-bisphosphate stimulates alveolar epithelial fluid clearance in male and female adult rats. Am J Physiol Lung Cell Mol Physiol 301: L804–L811, 2011. First published August 26, 2011; doi:10.1152/ajplung.00445.2010.— Cell membrane phospholipids, like phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2], can regulate epithelial Na channel (ENaC) activity. Gender differences in lung ENaC expression have also been demonstrated. However, the effects in vivo on alveolar fluid clearance are uncertain. Thus PI(4,5)P2 effects on alveolar fluid clearance were studied in male and female rats. An isosmolar 5% albumin solution was intrapulmonary instilled; alveolar fluid clearance was studied for 1 h. Female rats had a 37 ± 19% higher baseline alveolar fluid clearance than male rats. Bilateral ovariectomy attenuated this gender difference. Compared with controls, Pi(4,5)P2 instillation (300 μM) increased alveolar fluid clearance by ~93% in both genders. Amiloride or the specific eENaC small-interfering RNA inhibited baseline and Pi(4,5)P2-stimulated alveolar fluid clearance in both genders, indicating a dependence on amiloride-sensitive pathways. The fraction of amiloride inhibition was greater in P(4,5)P2-instilled rats (male: 64 ± 10%; female: 70 ± 11%) than in controls (male: 30 ± 6%; female: 44 ± 8%). Pi(4,5)P2 instillation lacked additional alveolar fluid clearance stimulation above that of terbutaline, nor did propranolol inhibit alveolar fluid clearance after Pi(4,5)P2 instillation, indicating that Pi(4,5)P2 stimulation was not secondary to endogenous β-adrenoceptor activation. Pi(4,5)P2 amine instillation resulted in an intermediate alveolar fluid clearance stimulation, suggesting that, to reach maximal alveolar fluid clearance stimulation, Pi(4,5)P2 must reside in cell membranes. In summary, Pi(4,5)P2 instillation upregulated in vivo alveolar fluid clearance similar to short-term β-adrenoceptor upregulation of alveolar fluid clearance. Pi(4,5)P2 stimulation was mediated partly by increased amiloride-sensitive Na transport. There exist important gender-related effects suggesting a female advantage that may have clinical implications for resolution of acute lung injury.

Acute lung injury; alveolar epithelial barrier; amiloride; β-adrenoceptor agonists; sodium transport; phosphatidylinositol-4,5-bisphosphate; pulmonary edema; female advantage in alveolar fluid clearance

Alveolar fluid clearance is driven by active Na transport across the alveolar epithelial barrier (29, 31, 33, 34). Several studies have demonstrated that exogenous administration of β-adrenoceptor agonists can stimulate alveolar fluid clearance (4, 31, 33, 39). In addition, endogenous catecholamine release increases alveolar fluid clearance by β-adrenoceptor stimulation (12, 31, 35). However, catecholamine-independent pathways can also increase alveolar fluid clearance, such as growth factors (5, 15, 46).

Phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] is a normal component of the inner leaflet of the plasma membrane, and recent work demonstrated that polypehosphoinositides {i.e., PI(4,5)P2 and phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P3]} are able to interact directly and indirectly with the β- and γ-subunits of the amiloride-sensitive epithelial Na channel (ENaC) (27, 36). Those studies were all based on electrophysiological measurements of ENaC, and, while the studies showed that addition of exogenous PI(4,5)P2 and PI(3,4,5)P3 affects ENaC channel function, these results did not directly prove that a physical interaction between the phosphatidylinositol phosphates and ENaC subunits occurs in vivo. Several studies have suggested that ENaC is responsible for maintaining dry alveoli (31) and is critical for the conversion from fluid secretion to absorption at birth (19). Data suggest that β-adrenoceptor stimulation of alveolar fluid clearance is a plausible route for treatment of pulmonary edema in patients recovering from acute respiratory distress syndrome (30). Recently, it has been shown both in isolated alveolar epithelial type I and type II cells and in lung slices that both cell types express ENaC, cystic fibrosis transmembrane receptor, and basolateral Na-K-ATPases needed for transepithelial ion transport (6, 11, 20). Most of the ENaC-Pi(4,5)P2 interaction studies suggest that Pi(4,5)P2 regulates ENaC by increasing its open probability (25, 37), while some more recent studies go further and claim that Pi(4,5)P2 is even necessary for ENaC activity to be maintained (26). There is little known about gender differences in relation to alveolar fluid clearance in the lungs. Sweezey and colleagues (43) demonstrated that female rats had a higher lung ENaC mRNA expression than male rats, suggesting that female rat’s capacity to clear the alveolar spaces from excess fluid would be greater. Zeitzlin and colleagues (49) demonstrated that the presence of female gender hormones to cultured airway epithelial cells altered the amount of short-circuit current that was inhibitable by amiloride. Additionally, recent work by Bastarache and colleagues (3) for the first time evaluated alveolar fluid clearance in male and female patients in a clinical setting and showed that alveolar fluid clearance is faster in women with acute lung injury compared with men. To our knowledge, there has been no systematic undertaking to determine if these differences also translate to an increased alveolar fluid clearance mediated by ENaC in female subjects compared with male subjects.

Therefore, we first tested the hypothesis that alveolar fluid clearance could be upregulated by phospholipids in the cell membrane.
membrane. The second objective was to determine whether there were gender-specific differences in alveolar fluid clearance with and without PI(4,5)P_2. The third objective was to determine if the fractional inhibition of alveolar fluid clearance by amiloride changed after PI(4,5)P_2 instillation in both female and male rats. The fourth objective was to determine if the increased alveolar fluid clearance after PI(4,5)P_2 instillation was sensitive to β-adrenoceptor stimulation or secondary to endogenous catecholamine release in both female and male rats. The fifth objective of these studies was to investigate whether cytoplasmic presence was sufficient to stimulate alveolar fluid clearance by using a PI(4,5)P_2 amine compound lacking the principal membrane binding parts in both female and male rats.

METHODS

Animals, surgical preparation, and ventilation. Male Sprague Dawley rats (n = 98) weighing 300–350 g and female Sprague Dawley rats (n = 63) weighing 275–325 g (Harlan Laboratories, Indianapolis, IN) were housed at a 12:12-h day-night rhythm and had free access to standard rat chow (Purina, Copley Feed, Copley, OH) and tap water. In addition, eight ovariectomized (OVX) rats were purchased from Harlan Laboratories. These rats were housed under the same conditions as the normal rats. The Institutional Animal Care and Use Committee at the Northeastern Ohio Universities Colleges of Medicine and Pharmacy (Rootstown, OH) has reviewed and approved the studies.

The rats were anesthetized with 50 mg/kg body wt of pentobarbital sodium (Nembutal; Abbott Laboratories, Chicago, IL) intraperitoneally. A 0.2 mm ID PE-240 (Clay Adams, Becton Dickinson, Parsippany, NJ) endotracheal tube was inserted through a tracheostomy. A PE-50 catheter (Clay Adams, Becton Dickinson) was inserted in the right carotid artery to monitor systemic blood pressure and to obtain blood samples. Pancuronium bromide (0.3 mg/kg body wt; Sicor Pharmaceuticals, Irvine, CA) was given intravenously hourly for neuromuscular blockade. The rats were maintained in the left lateral decubitus position during the experiments. The rats were ventilated with a constant-volume piston pump (Harvard Apparatus, Dover, MA) with an inspired oxygen fraction of 1.0 and with peak airway pressures of 7–9 cmH_2O during the baseline period, supplemented with a positive end-expiratory pressure of 4 cmH_2O.

Preparation of the instillates and other solutions. PI(4,5)P_2 (L-α-phosphatidylinositol-4,5-bisphosphate, trimannium salt; brain origin; porcine) was purchased from Avanti Polar Lipids (Birmingham, AL) and used as received. The PI(4,5)P_2 was dissolved in chloroform, methanol, and water in a 20:9:1 volume ratio. The mass was checked by weighing the vial before and after lipid solubilization using a Sartorius semimicro balance. The stock solution was divided to make PI(4,5)P_2 solution with or without 300 μM BSA (Sigma). Alveolar fluid clearance was determined by using the following mass balance equation: AFC = Albf(100) - Albi, where alveolar fluid clearance (AFC) was expressed as the percentage of instilled liquid that left the airspaces during the 1-h observation period, and Albi and Albf are the initial and final instillate protein concentrations, respectively. These solutions were always freshly prepared at room temperature within 30 min of use and had an average osmolality of 113 ± 10 mosmol/kgH_2O, irrespective of pDNA concentration, measured by a Vapor Pressure Osmometer 5500 (Wescor, Logan, UT). The pDNA was administered using a tracheal instillation procedure mainly according to Folkesson and colleagues (16). In short, during a brief isofluorane anesthesia, the rats were placed on a slanted board (20° from vertical) hanging from their upper incisors. The prepared pDNA/Lipofectamine solution was then delivered via the mouth to the lungs using a modified syringe needle in a volume of 1.5 ml/kg body wt. After the instillation, the rats were allowed to recover in their respective cages where they remained for the 24-h study.

General experimental protocol. In all experiments, after surgery, a 30-min baseline of stable heart rate and blood pressure was required before fluid instillation. An instillation tubing (PE-50 catheter; Clay Adams, Becton Dickinson) was gently passed through the tracheal tube and into the lung. Next, 3 ml/kg body wt of fluid (instillate) was instilled over 25 min into the lung for the 1-h studies. The 1-h studies began at the start of instillation of the fluid. The fluid was instilled by instilling 0.04 ml/min using a 1-ml syringe. After instillation, the tubing was withdrawn.

At the end of the experiments, a blood sample was obtained, the abdomen was opened, and the rats were exsanguinated by transecting the abdominal aorta. The lungs were removed through a median sternotomy. An alveolar fluid sample (0.1–0.2 ml) was obtained by gently passing the sampling catheter (PE-50 catheter; Clay Adams, Becton Dickinson) into a wedged position in the instilled area of the right lung. We previously reported that liquid aspirated with a catheter wedged in the distal air spaces is a good reflection of alveolar fluid protein concentration (33). Albumin concentrations in the instillates and aspirates were determined by refractometry. The refractometer (American Optical, Buffalo, NY) was calibrated using a series of albumin standards (Sigma). Alveolar fluid clearance was determined using the following mass balance equation: AFC = (1 – Alb/Alb)(100), where alveolar fluid clearance (AFC) was expressed as the percent of instilled liquid that left the airspaces during the 1-h observation period, and Alb and Alb are the initial and final instillate albumin concentrations, respectively. The term alveolar fluid clearance does not, however, imply that all reabsorption of fluid occurs at the alveolar level; some liquid reabsorption may occur across distal bronchial epithelium (2).

Specific treatments are summarized in Table 1. Hemodynamics and airway pressure. Peak airway pressure, arterial blood pressure, and heart rate were measured with calibrated pressure transducers (ADInstruments, Colorado Springs, CO) connected to analog-to-digital converters and amplifiers (MacLab/QUAD Bridge and MacLab/8, respectively; ADInstruments) and continuously recorded with Chart version 4.2.4 software (ADInstruments) on an IBM PC-compatible computer.
Table 1. Breakdown of the different treatments and number of animals per treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Male</th>
<th>Female</th>
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<tbody>
<tr>
<td>Control</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>OVx</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>PIP2 (150 μM)</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>PIP2 (300 μM)</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>PIP2 (750 μM)</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Am + PIP2</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>pSi-0 + PIP2</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>pSi-4 + PIP2</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Ter + PIP2</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Prop + PIP2</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>PIP2-Amine</td>
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<td>6</td>
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OVx, ovariectomized rats; PIP2, 1-α-phosphatidylinositol-4,5-bisphosphate; Am, ameloride (10 –3 M); pSi-0, control plasma DNA treatment; pSi-4, α-epithelial Na channel knockdown treatment; Ter, terbutaline (10 –4 M); Prop, propranolol (10 –3 M).

Plasma gender hormone measurements. Plasma 17β-estradiol concentrations were measured by an enzyme-linked immunosorbent assay (Cayman Chemical, Ann Arbor, MI). The inter assay and intra-assay variabilities for the 17β-estradiol assay were 6.3 and 9.7%, respectively.

Statistics. All data are summarized and presented as means ± SE. The data were analyzed by (1- and 2-way) ANOVA with Tukey’s post hoc test, or Student’s t-test when comparing the significance of two means such as the evaluation of relevance in male vs. female baseline alveolar fluid clearance values. Percent increase or inhibition of two means such as the evaluation of relevance in male vs. female baseline alveolar fluid clearance was calculated as (AFCy – AFCx)/AFCx, where, e.g., x is baseline female alveolar fluid clearance and y is baseline male alveolar fluid clearance resulting in the percent increase in alveolar fluid clearance. Standard errors are calculated according to standard error analysis procedures.

RESULTS

Dose-response relationship between PI(4,5)P2 concentration and stimulation of alveolar fluid clearance. To determine the best dose of PI(4,5)P2 for these studies, a dose-response relationship was generated by instilling male rats with 0–750 μM PI(4,5)P2 (Fig. 1A). From this dose-response relationship, we selected 300 μM PI(4,5)P2 as the dose we used for the main studies. We also normalized the alveolar fluid clearance stimulation by PI(4,5)P2 against the maximal stimulation of alveolar fluid clearance from the PI(4,5)P2 instillation (Fig. 1B). From that curve, we then determined the EC50 value for in vivo PI(4,5)P2 stimulation of alveolar fluid clearance and found it to be 170 μM.

Alveolar fluid clearance in male and female rats and effect of PI(4,5)P2. There were no changes in arterial blood gases, systemic blood pressure, and airway pressure after fluid instillation in the PI(4,5)P2-instilled compared with control rats in the 1-h studies in either gender.

We tested for gender differences in alveolar fluid clearance and found that baseline alveolar fluid clearance was significantly higher (absolute increase of alveolar fluid clearance in female rats is 4.4 ± 1.1% corresponding to a rate increase of 37 ± 19%; P < 0.05; Student’s t-test) in female rats than in male rats (Fig. 2A). PI(4,5)P2 (300 μM) instillation resulted in significant increases in alveolar fluid clearance over 1 h compared with control rats in both male and female rats (Fig. 2B). The percent increase in alveolar fluid clearance by PI(4,5)P2 in both male and female rats appeared similar (male: 104 ± 14%; female: 80 ± 13%), thus resulting in higher absolute alveolar fluid clearance levels in female rats than in male rats (Fig. 2B).

To determine the importance of the gender hormone estrogen, we carried out two sets of studies; first, we measured baseline alveolar fluid clearance in OVx rats, and, second, we measured plasma concentrations of 17β-estradiol in female control and OVx rats as well as in male control rats. The elevated baseline alveolar fluid clearance observed in female rats was attenuated in the OVx rats (Fig. 2C). Plasma concentrations of 17β-estradiol were reduced significantly in the OVx female rats compared with the female control rats (Fig. 2D). Male rats showed the same minimal (baseline) 17β-estradiol levels in their plasma as the OVx females (Fig. 2D).
Effect of amiloride on PI(4,5)P₂-stimulated alveolar fluid clearance in male and female rats. To determine whether the increase in alveolar fluid clearance from PI(4,5)P₂ (300 µM) instillation and gender depended on increased Na uptake by lung epithelial cells, amiloride (10⁻³ M) was added to the 5% albumin instillate and instilled in the distal air spaces of the rats. We also determined whether the fractional amiloride inhibition was different between control and PI(4,5)P₂-stimulated male and female rats. Amiloride inhibited the increase in alveolar fluid clearance from the distal air spaces in both control and PI(4,5)P₂-stimulated rats (Fig. 3, A and B). Amiloride-sensitive alveolar fluid clearance in male rats increased from 3.55 ± 0.75 to 15.5 ± 2.2% (P < 0.05; Student’s t-test) and in female rats from 7.1 ± 1.2 to 20.1 ± 3.0% (P < 0.05; Student’s t-test) upon addition of PI(4,5)P₂. Interestingly, amiloride-insensitive alveolar fluid clearance was found to be constant at ~8.8% (Fig. 3, A and B). These results translate into a fractional inhibition by amiloride that was significantly greater in PI(4,5)P₂-stimulated rats (66 ± 15%) than in control rats (37 ± 10%; P < 0.05; Student’s t-test) (Fig. 3, A and B). Amiloride seemed to inhibit more of the alveolar fluid clearance in female rats than in male rats both with (male: 64 ± 10%; female: 70 ± 11%; P < 0.05; Student’s t-test) and without (male: 30 ± 6%; female: 44 ± 8%; P < 0.05; Student’s t-test) PI(4,5)P₂ (Fig. 3, A and B). As a confirmation that we inhibited ENaC, we also knocked down ENaC expression in male rats with specific small-interfering RNA (siRNA) against αENaC (data not shown). Knockdown results were consistent with our previous results (23). After αENaC knockdown, alveolar fluid clearance was similarly inhibited as after amiloride instillation [control: 30 ± 18%; PI(4,5)P₂: 66 ± 14%; P < 0.05; Student’s t-test], thus supporting that amiloride inhibits ENaC (Fig. 3C).

Effect of terbutaline and propranolol instillation on PI(4,5)P₂-stimulated alveolar fluid clearance in male and female rats. The addition of the β₂-adrenoceptor agonist terbutaline to the instillate of the male and female control rats increased alveolar fluid clearance by ~112 ± 18%, not significantly different from that after PI(4,5)P₂ (300 µM) instillation (Fig. 4A). It seemed that the terbutaline stimulation of alveolar fluid clearance in female rats was lower (80 ± 12%) than in the

**Fig. 2.** A: alveolar fluid clearance expressed as %instilled volume in normal control male and female rats. Values are means ± SE; *P < 0.05 compared with male rats; Student’s t-test. B: alveolar fluid clearance expressed as %instilled volume in PI(4,5)P₂-instilled (300 µM) male and female rats. Values are means ± SE; *P < 0.05 compared with male control (Ctrl) rats; †P < 0.05 compared with female control rats; ‡P < 0.05 compared with male PI(4,5)P₂-instilled rats; ANOVA with Tukey’s post hoc test. C: alveolar fluid clearance expressed as %instilled volume in control female rats and in female ovariectomized (OVx) rats. Values are means ± SE; *P < 0.05 compared with female control rats; Student’s t-test. D: plasma 17β-estradiol concentrations in female control rats (n = 18), female OVx rats (n = 9), and male control rats (n = 8).

**Fig. 3.** A: alveolar fluid clearance expressed as %instilled volume in control male and female rats with and without 10⁻³ M amiloride (Amil). Values are means ± SE; *P < 0.05 compared with male control rats; †P < 0.05 compared with female control rats; ANOVA with Tukey’s post hoc test. B: alveolar fluid clearance expressed as %instilled volume in PI(4,5)P₂-instilled (300 µM) male and female rats and with and without 10⁻³ M amiloride. Values are means ± SE; *P < 0.05 compared with PI(4,5)P₂: 66 ± 14%; P < 0.05; Student’s t-test) PI(4,5)P₂ (Fig. 3, A and B). As a confirmation that we inhibited ENaC, we also knocked down ENaC expression in male rats with specific small-interfering RNA (siRNA) against αENaC (data not shown). Knockdown results were consistent with our previous results (23). After αENaC knockdown, alveolar fluid clearance was similarly inhibited as after amiloride instillation [control: 30 ± 18%; PI(4,5)P₂: 66 ± 14%; P < 0.05; Student’s t-test], thus supporting that amiloride inhibits ENaC (Fig. 3C).

Effect of terbutaline and propranolol instillation on PI(4,5)P₂-stimulated alveolar fluid clearance in male and female rats. The addition of the β₂-adrenoceptor agonist terbutaline to the instillate of the male and female control rats increased alveolar fluid clearance by ~112 ± 18%, not significantly different from that after PI(4,5)P₂ (300 µM) instillation (Fig. 4A). It seemed that the terbutaline stimulation of alveolar fluid clearance in female rats was lower (80 ± 12%) than in the
male rats (143 ± 13%; P < 0.05; Student’s t-test) (Fig. 4A). However, addition of terbutaline to PI(4,5)P2-instilled male and female rats did not result in an additional increase in alveolar fluid clearance (Fig. 4B) compared with normal rats instilled with terbutaline. The addition of the β-adrenoceptor antagonist propranolol to the instillate of the male and female control rats did not affect alveolar fluid clearance (Fig. 4C) nor did addition of propranolol to PI(4,5)P2-instilled rats affect alveolar fluid clearance (Fig. 4D). Also, there were no differences between male and female rats in response to propranolol either with or without PI(4,5)P2-stimulated alveolar fluid clearance (Fig. 4, C and D).

Effect of the PI(4,5)P2 amine on alveolar fluid clearance in male and female rats. To determine if membrane residency was more or less important for the ability of PI(4,5)P2 to stimulate alveolar fluid clearance in male and female rats, we instilled another group of male and female rats with the PI(4,5)P2 amine compound. Alveolar fluid clearance was stimulated to an intermediate level between PI(4,5)P2 (300 μM; full stimulation) and control (no stimulation) by the instillation of the PI(4,5)P2 amine compound (300 μM) in both male and female rats (Fig. 5). The absolute amount of alveolar fluid clearance in males and females after PI(4,5)P2 amine instillation was very similar (males: 6.8 ± 1.9%; females: 6.2 ± 2.4%), indicating that membrane residency is required for the full effect of PI(4,5)P2.

DISCUSSION

Phospholipids have been implicated in stimulating transepithelial Na transport and potentially alveolar fluid clearance in the lungs (27, 36), but no studies have so far investigated whether PI(4,5)P2 functionally can stimulate alveolar fluid clearance in the adult lung in vivo. The results of these studies in adult rats demonstrated a marked dose-dependent upregulation in alveolar fluid clearance following PI(4,5)P2 instillation

Fig. 4. A: alveolar fluid clearance expressed as %instilled volume in control male and female rats with and without 10⁻⁴ M terbutaline (Terb). Values are means ± SE; *P < 0.05 compared with male control rats; †P < 0.05 compared with female control rats; ANOVA with Tukey’s post hoc test. B: alveolar fluid clearance expressed as %instilled volume in male and female rats with 10⁻⁴ M terbutaline instilled with and without PI(4,5)P2 (300 μM). Values are means ± SE. C: alveolar fluid clearance expressed as %instilled volume in control male and female rats with and without 10⁻⁴ M propranolol (Prop). Values are means ± SE; *P < 0.05 compared with male control rats; ANOVA with Tukey’s post hoc test. D: alveolar fluid clearance expressed as %instilled volume in PI(4,5)P2-instilled (300 μM) male and female rats with and without 10⁻⁴ M propranolol. Values are means ± SE; *P < 0.05 compared with male PI(4,5)P2-instilled rats; †P < 0.05 compared with male PI(4,5)P2-propranolol-instilled rats; ANOVA with Tukey’s post hoc test.

Fig. 5. Alveolar fluid clearance expressed as %instilled volume in control male and female rats with and without 300 μM PI(4,5)P2 amine and with and without 300 μM PI(4,5)P2. Values are means ± SE; *P < 0.05 compared with male control rats; †P < 0.05 compared with female control rats; #P < 0.05 compared with male PI(4,5)P2 amine-instilled rats; ‡P < 0.05 compared with female PI(4,5)P2 amine-instilled rats; ANOVA with Tukey’s post hoc test.
(300 μM). In addition, there was a higher alveolar fluid clearance in female rats than in male rats after 300 μM PI(4,5)P₂ instillation because of the higher baseline alveolar fluid clearance in female rats. The elevated baseline alveolar fluid clearance in the female rats was associated with the gender hormone estrogen. No further stimulation by β-adrenoceptor agonists was observed in either gender, nor was the 300 μM PI(4,5)P₂ stimulation blocked by instillation of β-adrenoceptor antagonists. Amiloride instillation inhibited 30–40% of baseline alveolar fluid clearance in male and female rats, confirming previous results (29, 31). The inhibition by amiloride rose to 64 ± 10% inhibition in male rats and to 70 ± 11% inhibition in female rats after 300 μM PI(4,5)P₂ instillation. Confirmatory siRNA knockdown studies of αENaC demonstrated similar results as after amiloride instillation with (66 ± 14% inhibition) and without (30 ± 18% inhibition) 300 μM PI(4,5)P₂. The results of the studies using the PI(4,5)P₂ amine compound (300 μM) suggested that PI(4,5)P₂ take up and residency in the cell membrane was necessary if PI(4,5)P₂ was to fully stimulate alveolar fluid clearance in the lungs.

First, we wanted to study if there existed a dose-response relationship in PI(4,5)P₂-stimulated alveolar fluid clearance in normal healthy rats and to determine the EC₅₀ for this dose-response relationship. We found an excellent dose-response relationship between the instilled PI(4,5)P₂ concentration and the alveolar fluid clearance response. After normalizing the alveolar fluid clearance values to percent of maximal stimulation of alveolar fluid clearance, we calculated the PI(4,5)P₂ EC₅₀ concentration to be 170 μM. Based on the results from these observations, we selected the dose of 300 μM PI(4,5)P₂ for use in these studies of PI(4,5)P₂ stimulation of alveolar fluid clearance. We decided to use this dose, since it gave near-maximal alveolar fluid clearance stimulation. However, this strategy may have faltered in that, in the combination studies with the β₂-adrenoceptor agonist terbutaline, an additive PI(4,5)P₂ stimulation of alveolar fluid clearance may have been overshadowed by the β-adrenoceptor stimulation.

Our principal objective of these studies though was to investigate if PI(4,5)P₂-stimulated alveolar fluid clearance in normal healthy rats and to determine the amiloride sensitivity of the observed alveolar fluid clearance changes. We found that PI(4,5)P₂ similarly increased alveolar fluid clearance in both genders and that it was sensitive to amiloride inhibition. Amiloride inhibited most (66 ± 10%) of the PI(4,5)P₂-stimulated increase in alveolar fluid clearance, in contrast to inhibiting 37 ± 7% of baseline alveolar fluid clearance. Thus, most of the PI(4,5)P₂-stimulated increase in alveolar fluid clearance probably occurred by increasing Na uptake through amiloride-sensitive channels in the alveolar epithelial cells. In normal rats, the amiloride inhibition (37 ± 7%) of alveolar fluid clearance represented values similar to the fractional inhibition that has been reported previously (29, 31). After PI(4,5)P₂ (300 μM) instillation, this amiloride-inhibitable fraction was increased, thus suggesting a modest upregulation of the activity of the amiloride-sensitive Na transport pathways, i.e., ENaC. Also, the time required for the stimulatory effect by PI(4,5)P₂ suggests that the stimulation occurred by an increased activation of these ion channels, e.g., ENaC. Most likely, PI(4,5)P₂ increased the activity of the ENaC channels in the cell membrane, as has been shown earlier in vitro (25–27, 37, 38, 47).

Thus, it is likely that an upregulated Na transport capacity across the alveolar epithelium was the mechanism responsible for the increased alveolar fluid clearance after PI(4,5)P₂ instillation. Our data also suggest that it is unlikely that the upregulated alveolar fluid clearance was mediated by increases in ENaC expression, since we found no treatment-specific differences in ENaC expression (as measured by total mRNA levels; data not shown). Thus, it is plausible that an increased capacity for transepithelial Na transport across the alveolar epithelium would be the main mechanism after PI(4,5)P₂ instillation. The data suggest also that an increase in ENaC function may be a significant component of the response to the 300 μM PI(4,5)P₂ instillation, since the fractional amiloride inhibition was greater after PI(4,5)P₂ instillation.

We also tested if there were significant gender differences in alveolar fluid clearance in these rat lungs and found that baseline alveolar fluid clearance was elevated by ~37% in female rats compared with male rats. This observation is in agreement with the data published by Sweezey and colleagues (43) where they demonstrated that female rats had a higher lung ENaC expression than male rats. Additionally, Bastarache and colleagues (3) recently showed that alveolar fluid clearance is faster in women with acute lung injury compared with men. The effects from the female gender hormones estrogen and progesterone were also studied by Sweezey and colleagues (43). The data from those studies suggested that it did not really matter whether the rats were in the estrogen phase or progesterone phase of their estrous cycle; they had elevated mRNA levels for ENaC in either part of the cycle compared with male rats. In our studies, when estrogen was taken out of the picture, the elevated control alveolar fluid clearance in the female rats was no different from that in the male control rats. These results suggest that females may have an advantage over males in clearing the lungs from pulmonary edema or when clearing fetal lung fluid at birth, related to the presence of estrogen. In fact, it has been noted that prematurely born human girls exhibit a lower incidence of respiratory distress syndrome than do boys (1). We then tested if this elevated baseline alveolar fluid clearance also translated into a higher stimulation by females than males when the rats were given 300 μM PI(4,5)P₂. Conversely to what was expected, female rats exhibited the same rate of stimulation as male rats; however, female rats had a higher absolute alveolar fluid clearance. Female rats also demonstrated an elevated amiloride sensitivity, which is what would be expected based on the observations by Sweezey and colleagues (43). These data confirm the observations by Sweezey and colleagues (43) and extend their data to the in vivo situation, and thus may explain the “female advantage” in recovering from respiratory distress syndrome and pulmonary edema.

Another objective of these studies was to test if instillation of PI(4,5)P₂ resulted in alveolar fluid clearance stimulation secondary to alterations of the responsiveness to β-adrenoceptor stimulation by 300 μM PI(4,5)P₂ instillation. We and other investigators have previously reported that β-adrenoceptor agonists, growth factors, and endogenous catecholamines were associated with an increased alveolar fluid clearance (31, 33, 35, 39, 46). It has also been suggested that ENaC possesses one or more phospholipid binding site(s), potentially making it sensitive to PI(4,5)P₂ regulation (25, 37, 38, 47, 50). We
investigated if the effects were secondary to endogenous epinephrine release and inhibited alveolar fluid clearance stimulation from the 300 μM PI(4,5)P2 instillation with a β-adrenoceptor antagonist, e.g., propranolol, and we also studied if addition of exogenous β2-adrenoceptor agonists, e.g., terbutaline, to PI(4,5)P2-instilled rats (300 μM) resulted in additional stimulation of alveolar fluid clearance. Propranolol did not affect the PI(4,5)P2-stimulated alveolar fluid clearance, so it is not likely that PI(4,5)P2 acted via endogenous epinephrine release. We investigated this possibility since several studies have reported that endogenous release of epinephrine can regulate alveolar fluid clearance (12, 22, 31, 35). Terbutaline, the β2-adrenoceptor agonist (10−4 M), and PI(4,5)P2 (300 μM) were nearly equally potent in stimulating alveolar fluid clearance in the rat; however, it did not result in an additive stimulation of alveolar fluid clearance in PI(4,5)P2-instilled rats.

In several species, a significant fraction of the stimulatory effect from β-adrenoceptor agonists on alveolar fluid clearance can also be inhibited by amiloride (31, 33). However, since both basal and stimulated alveolar fluid clearance by β-adrenoceptor agonists are inhibited similarly by amiloride, the data indicate that β-adrenoceptor agonists increase alveolar fluid clearance by stimulation of both amiloride-sensitive and amiloride-insensitive pathways. The amiloride insensitivity may be related in part to cation channels that are not inhibited by amiloride. In fact, recent data (8, 40) suggest the existence of a rod-type cyclic nucleotide-gated cation channel in the alveolar epithelium that could be involved in lung fluid movement. However, in this study, the results suggest that PI(4,5)P2 instillation (300 μM) affects the amiloride-sensitive fraction of alveolar fluid clearance more than it affects the amiloride-insensitive fraction. These results, together with the time needed after PI(4,5)P2 instillation for an effect, indicate that the amiloride-sensitive ENaC may become more highly activated. Indeed, the amiloride-sensitive ENaC channels possess PI(4,5)P2-binding domains in their gene sequences that may allow this channel to be upregulated following PI(4,5)P2 instillation (25, 37, 38, 47, 50).

Could an increased aquaporin (AQP) expression in lung epithelium or endothelium result in the observed increases in alveolar fluid clearance after PI(4,5)P2 instillation? AQPs are present in the lung by both expression and functional studies (7, 9, 13, 14, 31, 32, 44, 46). Dexamethasone pretreatment over 48 h increased AQPI expression in developing rat lungs (21). It is not known if AQPS expression, which is expressed in the alveolar epithelial type I cells (9), is sensitive to PI(4,5)P2 stimulation. Even if that were the case, it is unlikely that the observed increase in alveolar fluid clearance after PI(4,5)P2 instillation could be explained by an increased AQP expression. Transepithelial water movement requires an osmotic driving force for water to cross the barrier either through the lipid bilayer, tight junctions, or through AQP water channels. This driving force is generated by transepithelial Na transport via the amiloride-sensitive and -insensitive pathways, as well as through the basolaterally located Na-K-ATPase (10). However, more recent studies using transgenic knockout mice suggest that AQPs are of limited importance for absorption of lung fluid since genetically modified mice lacking these AQPs survive birth without respiratory complications and grow normally to adulthood (41, 45). Thus, stimulation of isosmolar alveolar fluid clearance after PI(4,5)P2 instillation is more likely to depend on increased activity of ENaC channels and/or Na-K-ATPases, thus generating a greater osmotic force across the alveolar epithelium.

What is the physiological role for phospholipids like PI(4,5)P2 in the lung? One potential role could be to maintain the basal rate of ENaC activation and alveolar fluid clearance, since recent studies have proposed that PI(4,5)P2 may control ENaC baseline expression and activity (26, 38). Recent studies using excised inside-out patches have shown that PI(4,5)P2 actually maintains ENaC activity (27), indicating that a reduction in channel activity is caused by reduced membrane PI(4,5)P2 levels. This implies that PI(4,5)P2 may be hydrolyzed in the membrane and reduced in amount, thus causing a reduced ENaC activity. This may occur via activation of purinergic P2Y and epidermal growth factor receptors as well as by reduced phosphoinositide kinase (PI 3-kinase) activity, since PI 3-kinase inhibits ENaC by reducing membrane PI(4,5)P2 content (26, 37). PI(4,5)P2 may also upregulate membrane ENaC levels by G proteins, such as Rh small GTPases (42). Thus, ENaC may be regulated by PI(4,5)P2 both directly and via defined signaling pathways (26, 37).

In summary, PI(4,5)P2 instillation of rats increased the rate of alveolar fluid clearance. PI(4,5)P2 instillation affects the amiloride-sensitive fraction of alveolar fluid clearance to a greater degree than the amiloride-insensitive fraction. Importantly, PI(4,5)P2 needs to be situated in the cell membrane if PI(4,5)P2 would stimulate alveolar fluid clearance as concluded from the results of the studies with the PI(4,5)P2 amine compound. Pharmacological treatment with phospholipids like PI(4,5)P2, if they can be positioned in the cell membrane, may increase the basal transport capacity of the alveolar epithelium in patients with pulmonary edema.

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


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