Physiological effect of protein kinase C on ENaC-mediated lung liquid regulation in the adult rat lung

Benjamin Soukup, Audra Benjamin, Maria Orogo-Wenn, and Dafydd Walters

Division of Biomedical Sciences, St. George’s University of London, London, United Kingdom

Submitted 3 February 2011; accepted in final form 23 September 2011

Soukup B, Benjamin A, Orogo-Wenn M, Walters D. Physiological effect of protein kinase C on ENaC-mediated lung liquid regulation in the adult rat lung. Am J Physiol Lung Cell Mol Physiol 302: L133–L139, 2012. First published September 23, 2011; doi:10.1152/ajplung.00031.2011.—Tight control of lung liquid (LL) regulation is vital for pulmonary function. The aim of this work was to determine whether PKC activation is involved in the physiological regulation of LL volume in a whole lung preparation. Rat lungs were perfused with a modified Ringer solution, and the lumen was filled with the same solution without glucose. LL volume was measured during a control period and after modulating drugs were administered, and net LL transepithelial movement (Jv) was calculated. When the PKC activator PMA (10^{-5} M) and the Ca^{2+} ionophore ionomycin (10^{-6} M) were instilled into the lung together, Jv was significantly reduced (P = 0.03). This reduction was blocked by the PKC inhibitor chelerythrine chloride (10^{-5} M; P = 0.98). When PMA and ionomycin were added with the β-adrenergic agonist terbutaline, the terbutaline-induced increase in Jv was abolished. Addition of PMA and ionomycin with the epithelial Na^{+} channel (ENaC) blocker amiloride had no additional inhibitory effect. Together, these results suggest that PKC is likely to be involved in LL absorption, and the ability of PMA/ionomycin to block the terbutaline-induced increase in Jv suggests that the downstream target of PKC is ENaC.

calcium-dependent protein kinase C; ionomycin; phorbol 12-myristate 13-acetate

tight control of luminal lung liquid (LL) volume is essential for normal pulmonary function. An excess or deficiency of LL in the airway and the alveoli leads to pathology, so mechanisms to control LL volume are critical. In the airway, there is a dynamic balance between secretion and absorption of liquid (30), and some of the cellular mechanisms involved have been shown to be present in alveolar cells (13). Cl^{-} transport seems to underlie liquid secretion into the lumen, while Na^{+} transport has been shown to be essential for liquid absorption. The Na^{+} absorptive mechanisms have received more attention than the Cl^{-} secretory mechanisms, and the airway epithelium has been better studied than the alveolar barrier, although the latter contributes by far more surface area (by a ratio of ≥500:1, if not 850:1) (18).

The principal channel responsible for Na^{+} absorption is the epithelial Na^{+} channel (ENaC), which has been the focus of much research. In the perinatal period, activation of lung ENaC is very sensitive to epinephrine, which causes a dramatic reversal of net transepithelial liquid flow (Jv), thereby facilitating the change at birth from a prenatal secreting phenotype to an absorption phenotype (9, 17). Hummler et al. (15) demonstrated that α-ENaC-deficient (−/−) mice were unable to survive beyond 40 h after birth. These animals died from acute respiratory failure with liquid-filled lungs, providing conclusive evidence that ENaC is vital for the liquid absorption at birth. During postnatal life, there is a dynamic balance between the secretory and absorptive processes. Although liquid absorption predominates in the liquid-filled lung, a secretory mechanism persists and can be demonstrated to be present under certain circumstances (25). The mechanisms of ENaC modulation in adult lungs, especially in the alveolus, are poorly understood, but the lung’s ability to cope with physiological and environmental changes must depend on dynamic control of ENaC activity.

Several studies have demonstrated that PKC can alter the flux of ions across pulmonary epithelial cells. These studies can be divided into two broad groups: those that have shown an acute (within 60 min) regulation of liquid flux and those that show a longer-term effect. This distinction is important in consideration of the possible mechanisms governing liquid flux. In the acute system, PKC has been shown to inhibit the activity of ENaC in a variety of models and systems (1, 11, 16), but none of these models and systems measured Jv directly in the whole lung. If longer-term models are used to analyze the effect of PKC, a similar result is shown. Brief exposure of primary cultured alveolar epithelium to PMA (a PKC activator) results in downregulation of ENaC mRNA expression 4 h after initial exposure (36). This regulation of ENaC is clearly due to gene modulation; however, this mechanism is unlikely to underlie the short-term effect of PKC.

The aim of this study was to ascertain whether PKC is able to functionally modulate LL movement in an in situ preparation of the whole lung. Work in cell culture systems does not necessarily translate to in vivo models because of the complexity of intact epithelia, competing intracellular pathways, and influences from possible systemic controls. Defining the role of PKC in modulating ENaC function will lead to a greater understanding of the physiological mechanisms involved in LL homeostasis.

MATERIALS AND METHODS

Ethical approval. All experiments were performed under license from the United Kingdom Home Office in accordance with the Animals (Scientific Procedures) Act 1986.

Surgical preparation. A total of 54 male Wistar rats (190–490 g body wt) were obtained from Harlan (Oxon, UK). They were housed on site under a 12:12-h light-dark cycle with free access to food and water and temperature regulated at 17–19°C.

The animals were anesthetized by an injection (2.7 ml/kg ip) of 25% Hypnorm [fentanyl citrate (0.315 mg/ml) and fluanisone (10 mg/ml); Veta Pharmaceuticals, Leeds, UK] and 25% Hypnovel [midazolam (5 mg/ml); Roche, Welwyn, UK] in 50% water.

Address for reprint requests and other correspondence: D. Walters, c/o Div. of Biomedical Sciences (Mail Point L3A), St. George’s Univ. of London, London SW17 0RE, UK (e-mail: dwalters@sgul.ac.uk).
Surgery. After anesthesia had been achieved, the rat was laid supine on a heated (37°C) Perspex platform. A midline incision was made from the level of the diaphragm to the superior aspect of the neck. An incision was made caudal to the thyroid into the trachea, and a stainless steel intratracheal tube (1.5 cm long, 2 mm ID) was inserted and tied in place. The lungs were ventilated by a rodent ventilator (model 683, Harvard Apparatus, Holliston, MA) with normal room air at 6 cmH₂O peak pressure, 2 cmH₂O end-expiratory pressure, and 80 breaths/min with ~2 mL tidal volume.

The chest was opened from the clavicles to the xiphisternum to reveal the heart and lungs. To prevent clotting, 250 μl of heparin (5,000 U/ml; CP Pharmaceuticals, Wrexham, UK) were injected into the left ventricle. An incision was made into the right ventricle just proximal to the pulmonary artery, into which a cannula was inserted and tied in place. A second incision was made into the heart apex, and a cannula was inserted through the mitral valve and into the left atrium and tied in place. The animals died by cardiac arrest under general anesthesia.

A positive-displacement constant-flow peristaltic pump was used to recirculate the perfusate through the rat’s pulmonary vasculature. Perfusion pressure was monitored continuously, and the flow rate was set at the start of the experiment to give a pressure of 5–7 mmHg. At this point, ventilation was discontinued. Flow was checked during the experiment by timed collection of perfusate (average flow 10 ml/min, range 3–15 ml/min). A bubble trap was present in the circuit proximal to the pulmonary artery.

Perfusate. The perfusate was a modified Ringer solution comprising 117 mM NaCl, 2.68 mM KCl, 1.25 mM MgSO₄, 1.82 mM CaCl₂, 20 mM NaHCO₃, 5.55 mM d-glucose, 12 mM HEPES, and 3% BSA (VWR International, Poole, UK); 190 ml of the perfusate were placed in a temperature-controlled reservoir at a constant 38°C and oxygenated with 95% O₂:5% CO₂ to maintain pH 7.4.

Lung liquid. The chemical composition of the LL was exactly the same as that of the perfusate, except d-glucose was excluded, as it is not normally found on the apical side of the epithelium, and its presence would increase the LL absorption rate initially; however, as its concentration falls, the absorption rate would decline nonlinearly and make interpretation of absorption rates at later stages of the experiment difficult (2). The LL volume instilled was 10–15 ml/kg body wt. Blue Dextran (2 × 10⁶ Da, 35-nm molecular radius; Sigma-Aldrich, Poole, UK) was thoroughly mixed into the LL (50 mg/ml stock solution diluted 1:10 with LL) before instillation and acted as an impermeant tracer, allowing calculation of transepithelial LL movement from the change in its concentration over time. In some experiments, including the initial sample, were taken in each experiment; these latter samples constituted the test period. A total of 12 or 13 samples, including the initial sample, were taken in each experiment (Fig. 1).

At the end of the experiment, the lungs and ventricles were dissected out of the rat and weighed wet and then weighed again after several days in a 60°C oven until constant dry weights were achieved. LL analysis. The LL samples were centrifuged at ~800 g for 20 min at 20°C to ensure that contaminants, e.g., cells and debris, did not interfere with spectrometric readings.

A spectrophotometer (Ultrospec 3100 Pro, Biochrom, Cambridge, UK) was used to measure Blue Dextran concentration at 620 nm and vitamin B12 concentration at 360 nm. Corrections were made for the “spill” of absorption of Blue Dextran measured at 620 nm to measurements at 360 nm by including standards during each set of analyses. An increase in the Blue Dextran and vitamin B12 concentration indicated absorption of LL across the pulmonary epithelium.

The volume of accumulated LL can be calculated for any given point in time using the equation described by Walters and Oliver (32). The same calculation was made for vitamin B12, and any increase in the calculated volume from that for Blue Dextran would indicate an increase in the epithelial permeability. Samples were taken from the perfusate, and they too were analyzed for the presence of Blue Dextran and vitamin B12.

All results are expressed as Jₑ (in ml·min⁻¹·g dry lung wt⁻¹). Values are means ± SE; in all experiments, n = 6, except for ionomycin alone (n = 7) and terbutaline with PMA + ionomycin (n = 5). Paired t-test was used to determine statistical significance within experimental groups, and unpaired t-test was used to determine statistical significance between experimental groups. P ≤ 0.05 was the significance level for both tests.

RESULTS

PKC activation. PMA (10⁻⁵ M), when administered alone, produced no change in the resting LL absorption rate (P = 0.16; Fig. 2A), but when the Ca²⁺-ionophore ionomycin (10⁻⁶ M) was given in conjunction with PMA (10⁻⁵ M), LL absorption was significantly reduced (P = 0.03; Fig. 2B). Ionomycin
(10⁻⁶ M) alone increased LL absorption, from -0.016 ± 0.001 to -0.025 ± 0.002 ml·min⁻¹·g⁻¹ (P = 0.02).

**PKC inhibition.** Two PKC-specific inhibitors were used individually in separate experimental groups: chelerythrine chloride (10⁻⁶ M) and GF109203X (10⁻⁵ M; Fig. 3). Although both compounds increased the absorption rate when given alone, the increases were not statistically significant (P = 0.20 and P = 0.15, respectively).

Simultaneous administration of PMA (10⁻⁵ M), ionomycin (10⁻⁶ M), and chelerythrine chloride (10⁻⁶ M) resulted in no change in absorption rate compared with the control (P = 0.56; Fig. 4). This same result was obtained using the other PKC inhibitor, GF109203X (10⁻⁵ M; P = 0.98; Fig. 4). These results confirm that the PKA/ionomycin effect involved PKC.

**ENaC activation.** Activation of ENaC with the β-adrenergic agonist terbutaline [see data previously published by our group (34)] in the presence of PMA and ionomycin resulted in an inhibition of the increased absorption rate normally produced by terbutaline (Fig. 5A), so that there was no statistical difference between the control rate and the test rates (P = 0.12). The significant difference between the effects of terbutaline alone and the effects of administration of PMA + ionomycin with terbutaline (P = 0.02) supports the view that PKC activation inhibits ENaC function.

**ENaC inhibition.** We previously demonstrated that 5 × 10⁻⁵ M amiloride (an ENaC blocker) caused the cessation of LL absorption in our preparation (34). If PKC is acting via a different channel, one would expect it to have an additive blocking effect (which would produce net secretion of LL) when activated in conjunction with ENaC inhibition. The results confirmed that amiloride in the presence of PMA and ionomycin causes a significant reduction in Jv (P = 0.05; Fig. 5B). This effect was not significantly different from data previously published by our group (34) of the effect of amiloride alone on Jv. (P = 0.79). These data again suggest that the effect of PKC is likely to be mediated through ENaC.

**PMA and perfusion pressure.** PMA produced a steady rise in perfusion pressure over the 50 min of observation in all experiments in which it was administered (from 5.72 ± 0.16 mmHg in the control period to 15.51 ± 0.58 mmHg at the end of the experiment, P < 0.001, n = 35). This effect was not blocked by the two inhibitors of PKC, although they were able to block the effects on Jv.

Ionomycin alone had little effect on perfusion pressure, (from 6.38 ± 0.62 to 8.67 ± 1.30 mmHg, n = 7, P = 0.18,) and in all other experiments, perfusion pressure did not rise (from 5.94 ± 0.26 to 6.20 ± 0.28 mmHg, n = 21).

**Epithelial integrity.** Vitamin B12 was used to investigate whether PMA and/or the resulting increase in perfusion pressure affected the epithelial integrity of the lungs and, subsequently, our results. In 13 experiments where vitamin B12 and Blue Dextran were used together, there was no significant difference in any experiment between Jv obtained by vitamin B12 and Jv obtained by Blue Dextran. Analysis of perfusate samples taken throughout each experiment showed no Blue Dextran or vitamin B12 in the perfusate.
DISCUSSION

Understanding of the control of airway liquid volume has improved markedly in the past few years (10). It is thought that changes in the concentration of soluble molecules (e.g., nucleosides, nucleotides, and protease inhibitors) in the lining liquid are able to inhibit or activate anion and cation channels, allowing accurate and sensitive measurement of liquid volume and, thus, changes in \( J_v \) over a relatively short period.

**PKC activation.** PKC has been shown to inhibit \( \text{Na}^+ \) currents in a variety of epithelia. Ling and Eaton (16) showed a decrease in the open channel probability (\( P_o \)) of \( \text{Na}^+ \) channels in A6 cells to values near zero after only 15–30 min of exposure to the PKC activator PMA. Mohrmann et al. (20) showed a 50% inhibition of \( ^{22}\text{Na}^+ \) uptake in LLC-PK1 cells after 10 min of PMA treatment, and Awayda et al. (1) demonstrated inhibition of whole cell currents in *Xenopus* oocytes following 1 h of treatment with PMA. In contrast, Volk et al. (31) showed that activation of PKC by PMA increased ENaC activity in *Xenopus* oocytes after 10 min of exposure. Chelerythrine was able to significantly inhibit ENaC currents. In a study similar to the present study, Berthiaume et al. (5) demonstrated that addition of \( 10^{-7} \) M PMA to sheep lungs had no effect on LL clearance.

An in vitro study by Chen et al. (11) showed that the influenza virus is able to rapidly inhibit ENaC in type II rat alveolar culture cells. This effect could be blocked using the specific PKC inhibitor GF109203X. Chen et al. provided strong evidence that PKC does inhibit ENaC in the acute phase (within 60 min of viral exposure); furthermore, ENaC inhibition was shown to be due to PLC and Src activation of PKC. Chen et al. were not able to demonstrate any concurrent change in epithelial permeability. An interesting comparison can be made with the study in which PKC activity was measured following flooding of rat lungs (26). A rise in PKC activity was found, but only after 4 h postflooding, suggesting that PKC has a role in modulating LL absorption. In our study, we were able to demonstrate that, by directly stimulating PKC activity, a fall in LL absorption rate could be measured. These two previous findings show that PKC activation is involved in regulating LL, and our study shows that PKC activation has an immediate physiological effect.

Our results provide the first data in the whole lung of decreasing liquid absorption in response to PKC activation, an
action that is most likely due to its effect on ENaC. Previous studies have focused largely on single-cell patch-clamping techniques, which, by and large, have demonstrated that PKC inhibits ENaC in the acute phase.

**PMA and epithelial permeability.** The effect of PMA in our experiments does not appear to be due to a change in epithelial permeability, although this has been previously described (21, 35). Samples of perfusate taken during and at the end of the experiments were analyzed for the presence of Blue Dextran and vitamin B12, and neither tracer was found to be present. Furthermore, calculations of LL volume changes using both volume markers were identical, although the molecular sizes (and, thus, the diffusion coefficients) of the markers are very different. Blue Dextran has a range of sizes (\(\approx 2 \times 10^6\) Da) and an average molecular radius of 35 nm, while vitamin B12 has a molecular mass of 1,353 Da and a molecular radius of 0.84 nm, which is intermediate between the sizes of mannitol (0.42 nm) and inulin (1.4 nm). The diffusion coefficient of vitamin B12 is \(\approx 40\) times greater than that of Blue Dextran.

**Ionomycin.** We showed that activation of PKC with PMA and ionomycin caused a dramatic reduction in the rate of LL absorption. This 60% reduction in the LL absorption rate was seen only when PMA was used in conjunction with ionomycin. Ionomycin is a specific Ca\(^{2+}\) ionophore that causes a release of Ca\(^{2+}\) from intercellular stores via a mechanism similar to inositol trisphosphate. The finding that Ca\(^{2+}\) is required in our system supports the view that the Ca\(^{2+}\)-dependent classical PKC ([c]PKC) isoform is mediating the effect. Gobran et al. (14) demonstrated the presence of (c)PKC in alveolar rat type II cells by showing the presence of its subunits: \(\alpha, \beta, \beta_\text{II}, \gamma\). Silver et al. (28) showed a similar Ca\(^{2+}\)-dependent mechanism of PKC activation in rat cortical collecting ducts and a consequential reduction in Na\(^+\) channel activity. The requirement of Ca\(^{2+}\) to activate PKC may be a reason why Berthiaume et al. (5) did not see any changes in the LL absorption rate following addition of PMA alone.

Ionomycin alone caused a significant increase in \(J_\text{w}\), which is opposite to the effect when ionomycin is combined with PMA. The former effect is most likely due to the documented effect of Ca\(^{2+}\) on increasing liquid clearance in fetal guinea pig lung (22) and in cell culture (29). Conversely, Planes et al. (24) showed that, in cultured SV40-transformed rat alveolar type II cells, ionomycin (10\(^{-6}\) M, 15 min) caused a reduction in Na\(^+\)-K\(^+\)-ATPase activity, which would be expected to reduce \(J_\text{w}\) in our preparation. This is opposite to our finding and may be due to the complexity and interactions of the regulating mechanisms in the intact pulmonary epithelium.

Yamagata et al. (36) reported that (c)PKC was the most likely PKC isoform involved in the ENaC inhibition of LL absorption. They showed that the specific (c)PKC inhibitor GF109203X was able to block the effect of PMA on PKC expression in cultured rat epithelial cells. However, a degree of caution must be adopted when comparing the study of Yamagata et al. with our study. Yamagata et al. showed a time-dependent fall in \(\alpha, \beta, \gamma\)-ENaC mRNA expression following PMA treatment. They measured ENaC mRNA levels 4 h after exposure to PMA. Our experiments measured the effect of PMA over a 50-min period. However, both sets of experiments indicate the involvement of the (c)PKC isoform and suggest that it has a role in LL volume homeostasis. Our work is the first in situ whole lung model to suggest that the (c)PKC isoform is able to regulate LL absorption in an acute setting, i.e., within 50 min of exposure.

The observation that PMA alone had no effect on LL movement yet always produced an increase in perfusion pressure suggests that the two effects are independent, at least over the duration of exposure observed in our experiments. Probably different isoforms of PKC are involved in the two effects. It is known that PKC induces vasoconstriction in rabbit and mouse pulmonary vasculature (33) and increases endothelial permeability (27).

Measurement of PKC species, activated or otherwise, directly in the lung tissue of our preparation would be possible but difficult to interpret. It would be very difficult to isolate the pulmonary epithelium in a timely fashion to extract the relevant proteins from such a histologically complex structure as the whole lung. Such measurements could be undertaken in primary cultures of certain epithelial cells, but then biochemical changes in function could not be correlated directly to the natural epithelium of the intact lung.

**PKC inhibition.** Initially, we attempted to block PKC in the unstimulated lung using chelerythrine chloride, a potent, selective antagonist of the Ca\(^{2+}\)/phospholipid-dependent PKC. The results showed no significant change in the rate of LL absorption at rest, which led us to believe that (c)PKC may not be tonically active. We therefore activated PKC with PMA and ionomycin and then administered chelerythrine chloride. This treatment completely blocked the reduction in LL absorption rate seen after the addition of PMA and ionomycin alone.

A second PKC inhibitor, GF109203X, selective for the \(\alpha\)- and \(\beta\)-isoforms (IC\(_{50}\) = 0.0084 and 0.0180 \(\mu\)M), was also used. GF109203X had an effect almost identical to that of chelerythrine in abolishing the effect of PMA and ionomycin on \(J_\text{w}\).

These findings go a step further in showing that PMA is indeed acting exclusively via PKC and not via a separate mechanism. Had another non-PKC-mediated mechanism been implicated, the PMA effect would not have been blocked when a specific PKC inhibitor was given. This is an important observation, as PKC is known to have multiple nonspecific effects on membrane trafficking in different systems. Additionally, Chen et al. (11) showed that PKC does not increase epithelial permeability in rat type II alveolar cells, nor did we detect a change in epithelial integrity when we used two different-sized volume markers. A PKC-mediated increase in epithelial permeability would create an effect similar to ENaC inhibition. These two findings make it likely that PKC is indeed acting via a specific epithelial channel.

Chen et al. (11) showed that pretreatment of ATII cells with GF109203X for 60 min resulted in an increase in \(P_o\) of Na\(^+\) channels, suggesting tonic inhibition of ENaC by PKC. Our results showed a 56% increase in LL absorption with chelerythrine and a 107% increase with GF109203X, but these results were not significantly different from control LL absorption rates (\(P = 0.20\) and \(P = 0.15\), respectively). However, the increase in LL absorption is in the same direction as found by Chen et al. It may be that a longer exposure to GF109203X in our system would have been indicated, but our preparation is not reliable after \(\sim 140\) min.

**ENaC activation.** We postulated that ENaC may be the ultimate downstream target of (c)PKC. This view is based on several studies that showed a reduction in ENaC expression...
following exposure to PMA (1, 7, 37). Our model is somewhat different: we are looking at the acute functional effect of PKC activation, while most previous studies focused on longer-term downregulation of ENaC mRNA. Chen et al. (11) demonstrated a significant reduction in \( P_o \) of ENaC channels in cells treated with a PKC-activating influenza virus. However, this work was carried out on ATII cells in primary culture, and not in vivo.

\( \beta_2 \)-Adrenergic stimulation has been shown to produce liquid absorption in the fetus (9) and to increase postnatal LL absorption (6) and increase the \( P_o \) of ENaC in lung epithelium (12, 19). Functionally, this leads to a 200% increase in \( P_o \) and a twofold increase in \( P_o \) (3). Terbutaline is thought to upregulate ENaC activity via a \( \beta_2 \)-receptor-mediated rise in cAMP. cAMP has been shown to stimulate PKA-dependent ENaC subunit trafficking to the apical membrane and direct phosphorylation of \( \beta \)- and \( \gamma \)-ENaC subunits (4).

In our model, we have shown that terbutaline increased LL absorption by 157% (34). Administration of PMA and ionomycin to the lungs, together with \( \beta_2 \)-mediated activation of ENaC, significantly reduced \( J_\ell \), compared with administration of terbutaline (\( P = 0.017 \); Fig. 5). This suggests that PKC activation is interfering with the intracellular signaling cascade of terbutaline on ENaC or is activating a competing pathway involved in ENaC subunit trafficking.

We have demonstrated that PKC activation is able to block the effect of \( \beta_2 \)-adrenergic stimulation of ENaC in an in situ whole organ model. This observation strengthens the argument that PKC is indeed inhibiting ENaC currents and is not working through another mechanism, e.g., stimulating a liquid secretory pathway to produce our observed results. Our method measures net liquid movement, i.e., the balance between secretion and absorption into and out of the lung lumen.

Although our preparation is flooded with liquid and, thus, presumably maximally stimulating any local liquid control mechanisms on the epithelium, a further substantial increase in absorption is produced by \( \beta_2 \)-adrenergic stimulation. It seems that local and systemic control mechanisms are able to modulate LL volume.

**ENaC inhibition.** It is well documented that amiloride specifically blocks LL absorption (2, 23), as shown in our model when a \( \sim 102\% \) change (decrease) in LL absorption was observed after addition of amiloride (34). When PMA and ionomycin were given in conjunction with amiloride, no significant change in \( J_\ell \), compared with that observed with administration of amiloride alone was seen. In addition, there was no significant difference in the reduction of the LL absorption rates between PMA and ionomycin alone and amiloride alone (\( P = 0.331 \)). These data show that PKC activation is not additive to amiloride-mediated ENaC inhibition, and they support the conclusion that PKA-mediated PKC inhibition is indeed acting on ENaC. If PKC had been acting on a separate channel, one would expect a larger fall in LL absorption; i.e., it would be additive to the effect of amiloride.

**Conclusion.** The results presented here suggest that PKC has a physiological effect in the whole lung (with its integrated intact epithelium) in reducing LL absorption in adult rats. Inhibition of PKC with inhibitors specific to the \( \epsilon \)PKC isoform was able to inhibit the effect of PKC activation by PMA and ionomycin. This suggests that PKC is responsible for the downstream effect of PMA and ionomycin on LL flux.

Activation of ENaC by \( \beta_2 \)-agonists caused a dramatic increase in LL absorption. This increase was blocked when PKC was activated, once more suggesting its involvement in the control of an ENaC-dependent LL flux. When amiloride was used to inhibit ENaC function, no additive effect was seen with PKC activation.

The observations presented here agree with previous cell culture studies. It appears that PKC acutely inhibits ENaC function in the in situ adult rat lungs, which translates into a measurable physiological response.

**GRANTS**

This work was funded by the Dewey Bequest.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

Author contributions: B.S., A.R.B., and D.V.W. are responsible for conception and design of the research; B.S., A.R.B., M.C.O.-W., and D.V.W. performed the experiments; B.S., A.R.B., M.C.O.-W., and D.V.W. analyzed the data; B.S., A.R.B., M.C.O.-W., and D.V.W. interpreted the results of the experiments; B.S., A.R.B., and D.V.W. drafted the manuscript; B.S., A.R.B., and D.V.W. approved the final version of the manuscript; A.R.B. prepared the figures; A.R.B. and D.V.W. edited and revised the manuscript.

**REFERENCES**
