ENaC activity and expression is decreased in the lungs of protein kinase C-α knockout mice

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First published July 11, 2014; doi:10.1152/ajplung.00040.2014.—We used a PKC-α knockout model to investigate the regulation of alveolar epithelial Na⁺ channels (ENaC) by PKC. Primary alveolar epithelial type II (ATII) cells were subjected to cell-attached patch clamp. In the absence of PKC-α, the open probability (Pₒ) of ENaC was decreased by half compared with wild-type mice. The channel density (N) was also reduced in the knockout mice. Using in vivo biotinylation, membrane localization of all three ENaC subunits (α, β, and γ) was decreased in the PKC-α knockout lung, compared with the wild-type. Confocal microscopy of lung slices showed elevated levels of reactive oxygen species (ROS) in the lungs of the PKC-α knockout mice vs. the wild-type. High levels of ROS in the knockout lung can be explained by a decrease in both cytosolic and mitochondrial superoxide dismutase activity. Elevated levels of ROS in the knockout lung activates PKC-δ and leads to reduced dephosphorylation of ERK1/2 by MAP kinase phosphatase, which in turn causes increased internalization of ENaC via ubiquitination by the ubiquitin-ligase Nedd4-2. In addition, in the knockout lung, PKC-δ activates ERK, causing a decrease in ENaC density at the apical alveolar membrane. PKC-δ also phosphorylates MARCKS, leading to a decrease in ENaC Pₒ. The effects of ROS and PKC-δ were confirmed with patch-clamp experiments on isolated ATII cells in which the ROS scavenger, Tempol, or a PKC-δ-specific inhibitor added to patches reversed the observed decrease in ENaC apical channel density and Pₒ. These results explain the decrease in ENaC activity in PKC-α knockout lung.

protein kinase C-α; epithelial Na⁺ channels; alveoli; single channels; knockout mice; lung fluid balance

A thin layer of fluid on the apical surface of the alveolar epithelium is critical for efficient gas exchange in the lung. This fluid layer is maintained by a balance between passive fluid secretion into the airway lumen, driven by hydrostatic pressure and active reabsorption of fluid into the vascular space (11).

Active sodium (Na⁺) transport from the airspace, across the alveolar epithelium, and into the pulmonary interstitium is the major mechanism for regulating lung fluid balance. Epithelial Na⁺ channels (ENaC) are responsible for the majority of Na⁺ transport across the apical membranes of alveolar epithelial cells (11, 20). The Na⁺ that enters the cells is subsequently transported by the Na⁺/K⁺-ATPase out of the cell and into the interstitium and pulmonary capillaries. This active Na⁺ transport, via ENaC, creates an osmotic potential across the apical alveolar epithelium. This osmotic gradient is the driving force in the removal of water from the distal air spaces in the lung or alveolar fluid clearance (AFC). The application of amiloride, an ENaC-specific inhibitor, reverses the majority of AFC, indicating that ENaC is indeed central to maintenance of proper fluid balance in the lung (20).

ENaC can be regulated either by altering the time individual channels remain open (open probability, or Pₒ), or by modifying expression of functional sodium channels in the membrane (N). An important signaling molecule that regulates ENaC is protein kinase C (PKC). Activation of PKC has been shown to reduce ENaC activity in A6 cells, a renal cell line (32, 48), and principal cells from rat kidney (15); conversely, inhibition of PKC has been observed to increase ENaC Pₒ (32, 53). Our laboratory has recently shown that ENaC activity and expression are significantly increased in isolated split-open collecting duct tubules from PKC-α knockout mice (3). One prior report and our current work have shown that there is only one PKC isoform, α, present in principal cells (30, 43), whereas there are multiple PKC isofoms in the lung (49). This suggested that the mechanism for PKC regulation of ENaC in the lung could be quite different than in the kidney. Therefore, we made use of the same PKC-α knockout mouse model to examine the regulation of ENaC by PKC in alveolar epithelium.

MATERIALS AND METHODS

Animals. The original PKC-α knockout mice were acquired from the laboratory of Dr. Jeffery Molkentin at the University of Cincinnati (34), and a colony was established at Emory University. Control mice were bred by backcrossing PKC-α knockout mice with SV129 control animals for ten generations. Subsequently, the wild-type line was maintained as a control for the PKC-α knockout mice. Mice were kept on a 12-h:12-h light/dark cycle and fed standard laboratory chow with tap water ad libitum. All animal protocols were approved by the Emory Institutional Care and Use Committee.

Antibody production. Polyclonal antibodies for the extracellular domain of the α-subunit of ENaC (890) and the carboxyl terminal domains of the β-subunit (60) and γ-subunit (2,102) of ENaC were generated in White New Zealand rabbits by Bio-Synthesis, Lewisville, TX using constructs made in house as described by Bao et al. (3).

Evans blue protein assay. A solution comprised of 5% bovine serum albumin and 0.1 mg/ml Evan’s blue dye was tracheally instilled, as described previously (26), in PKC-α knockout mice and SV129 control mice. The Evan’s blue solution was extracted from the lung after 30 min. AFC was calculated using the formula:

\[
\text{AFC} = \frac{(\text{V}_i - 100)}{\text{V}_f} = \frac{100}{\text{V}_f}
\]

where \( \text{V}_i \) is the initial volume of instillate, and \( \text{V}_f \) is the final alveolar fluid volume. \( \text{V}_f = (\text{V}_f - \text{EB}_f) / \text{EB}_f \), where \( \text{EB}_f \) is the concentration of Evan’s blue-labeled albumin in the instilled solution, and \( \text{EB}_f \) is the final concentration of Evan’s blue in the alveolar fluid (19). Protein concentration was measured via spectrophotometry (Thermo Scientific, Wilmington, DE).
Wet/dry weight assay. Whole lungs from PKC-α knockout and SV129 animals were bleed out, extracted, and weighed immediately following removal (wet weight). Subsequently, the lungs were desiccated overnight, at 110°C, and reweighed (dry weight). The wet-to-dry ratio was determined by dividing the wet weight by the dry weight, which represents the amount of airway fluid and is a measure of the rate of AFC. A larger ratio indicates more fluid in the lungs initially (less AFC), which implies less sodium transport attributable to decreased ENaC activity, whereas a smaller ratio indicates dryer lungs (more AFC) because of increased sodium transport via ENaC.

Chemicals. Most chemicals were obtained from Sigma-Aldrich (St. Louis, MO) except for the pseudo-substrate PKC-δ inhibitor, δ-PKC (8–17), from AnaSpec (Fremont, CA), and the mitogen kinase phosphatase (MKP) inhibitor [(E)-2-Benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one] from EMD Millipore (Billerica, MA). This inhibitor is a cell-permeable cyclohexylamino-indenone compound that acts as an allosteric inhibitor against substrate binding-induced MAPK phosphatase activity of Dusp1/MKP1 and Dusp6/MKP3 by locking Dusp1 and Dusp6 in their low-activity conformation. The inhibitor effectively enhances cellular ERK phosphorylation in cells overexpressing human Dusp1 and Dusp6 (EC50 = 11.5 and 12.3 μM, respectively) (39).

Fluorescent labeling and detection of reactive oxygen species. Lung slices (125 μm) were prepared, as described previously (24), from wild-type and PKC-α knockout mice. Subsequently, the slices were covered and incubated in 10 μM dihydroethidium (DHE) with 10 μM Erythrina crista-galli lectin (ECL), an alveolar type I cell marker, for 30 min at 37°C. It has been reported that production of 2-hydroxyethylidium from DHE is an accurate reporter of superoxide production (12, 19, 24, 24, 51). After incubation, DHE fluorescence was analyzed using confocal microscopy at excitation/emission 520/610 nm (19), and data were quantified using Image J (an open-source image analysis program).

Determining superoxide dismutase activity. Superoxide dismutase (SOD) activity in both wild-type and PKC-α knockout lung was determined using the Superoxide Dismutase Assay Kit (Cayman Chemical, Ann Arbor, MI). Samples were analyzed using a plate reader at excitation/emission 440/460 nm.

SDS-PAGE and immunoblotting. Freshly isolated lungs, from PKC-α knockout and SV129 control animals, were minced and then washed once with 1× PBS and subsequently homogenized in tissue protein extraction reagent (Thermo Scientific) using an Omni TH homogenizer (Warrenton, VA). Tissue lysates were then centrifuged at 1,000 revolution/min, at 4°C, for 5 min. The supernatant was then sonicated twice, on ice, for 10 s. Lysate protein concentration was determined using the BCA protein assay (Thermo Scientific). Twenty-five micrograms of total protein were prepared in Laemmli sample buffer (Bio-Rad, Hercules, CA) and then loaded and resolved on 7.5% Tris-HCl polyacrylamide gels using the Criterion or Protean electrophoresis systems (Bio-Rad). The separated proteins were electrically transferred onto Immobilon-P transfer membranes (Millipore). The membranes were blocked in 5% wt/vol milk in 1× TBS with Tween (TBST, Bio-Rad), at room temperature for 1 h. The membranes were washed once with 1× TBST, and then incubated with primary antibody at a dilution of 1:1,000 in 5% wt/vol milk in 1× TBST overnight at 4°C. The membranes were washed three times with 1× TBST for 5-min intervals before being incubated with horseradish peroxidase-conjugated goat-anti-rabbit secondary antibody at a dilution of 1:5,000 in 5% wt/vol milk in 1× TBST. The membranes were incubated with SuperSignal Dura Chemiluminescent Substrate for 5 min before being developed using a Kodak Gel Logic 2200 Imager and Carestream Molecular Imaging software (Carestream Health, Rochester, NY). This method was used to detect ENaC subunits (with in house antibodies) (1, 48, 52), ERK1/2 (9102; Cell Signaling, Beverly, MA), phosphoERK1/2 (9101a, Cell Signaling), MARKCS (SC-6454; Santa Cruz Biotechnology, Santa Cruz, CA), and phospho-MARKCS (PA1–4629, Thermo Scientific). PKC-α was detected with an antibody from Cell Signaling (9375), and PKC-δ was detected using an antibody from Genetex (GTX61153; San Antonio, TX).

In situ biotinylation. A protocol developed by Frindt and Palmer for in situ biotinylation of rat kidney (13, 14) was modified for use in mouse lung. Mice were anesthetized by intraperitoneal injection of 50 mg/kg sodium pentobarbital diluted in sterile saline. The abdominal and chest cavity were opened, and the aorta was cut to allow for bleeding out. An incision was made in the left atrium to release pressure, and the lung was perfused with PBS until the lungs appeared white or blanched. PBS containing 0.5 mg/ml sulfoconcanimidyl-2-[biotinamido]-ethyl-1,3-dithiopropionate (sulfo NHS biotin; Pierce, Rockford, IL) was allowed to flow through the lung via the trachea, after which the reaction was quenched by instilling the lung for 25 min with PBS containing 25 mM Tris-HCl, replacing the 25 mM NaCl. Subsequently, whole biotinylated lungs, from both wild-type and PKC-α knockout mice, were extracted, homogenized, and lysed in a buffer containing 250 mM sucrose and 10 mM triethanolamine (pH 7.4). To separate the total membrane fraction, the supernatant was centrifuged at 4°C for 6 h at 18,000 g. The total membrane fraction was suspended in 150 μl lysis buffer, equally loaded onto streptavidin beads (high-capacity neutravidin agarose resin, Thermo Scientific), and incubated overnight. The unbound protein was removed from the beads by first washing three times with wash buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, and 1% Triton-X 100, pH 7.4), followed by a high-salt wash (wash buffer with 500 mM NaCl), then two no-salt washes (10 mM Tris, pH 7.4). Afterward, the bound protein was eluted in sample buffer with 0.5 M diithiothreitol. Biotinylated and unbiotinylated fractions, from both the wild-type and PKC-α knockout animals, were run on 7.5% Tris-HCl polyacrylamide gels, and probed using in-house antibodies for α, β, and γ ENaC (as described above).

Single-channel patch clamp. Primary alveolar epithelial type II (ATII) cells were isolated from PKC-α knockout and SV129 control mice, as previously described (7, 8). ATII cells were prepared on a permeable support before single-channel patch clamp, as previously described for patch clamp of cells in culture (23, 28). Briefly, a microelectrode was filled with physiological buffer and applied to a single cell using suction, until a >1 GΩ seal was formed. ENaC channels were identified by characteristic channel kinetics and the current-voltage relationship for the channel.

Data analysis and statistics. Data acquisition and analysis were performed as described previously (23). Data are reported as means ± SE. Statistical analysis was performed with SigmaPlot and SigmaStat software (Jandel Scientific, San Rafael, CA). Statistical significance was evaluated using t-test, z-test, or one-way ANOVA, as appropriate, and results were considered significant if P < 0.05.

RESULTS

Apical alveolar ENaC expression is decreased in protein kinase C-α knockout mice. We used PKC-α global knockout mice to investigate the effect of PKC-α on ENaC activity in the alveoli. In Western blots, PKC-α could be detected in whole lung lysates from wild-type mice but could not be detected in lung lysates from PKC-α knockout mice (Fig. 1A). Western blotting showed no discernable difference in whole lung ENaC expression between the wild-type and knockout mice (Fig. 1B). However, only ENaC at the surface of alveolar epithelial cells contributes to AFC, and ENaC can move from the membrane to an intracellular pool. Therefore, we used an in situ biotinylation technique (described in MATERIALS AND METHODS) to label proteins on the apical surface of the alveoli. After precipitating the biotin-labeled proteins from whole lung lysates, we resolved the precipitates on gels and probed for all three ENaC subunits (Fig. 1C). The quantity of all three subunits (α, β, and γ ENaC) was determined using an antibody from Cell Signaling (9375), and PKC-δ was detected using an antibody from Genetex (GTX61153; San Antonio, TX).
γ) at the apical membrane in the knockout mice was decreased compared with wild-type (Fig. 1D), showing that apical-membrane expression of ENaC is decreased in PKC-α knockout mice.

Sodium transport is decreased in the PKC-α knockout lung. We quantified physiological AFC using the wet:dry weight assay and the Evan’s blue assay (Fig. 2). For the wet:dry weight assay, a higher value indicates more fluid in the lung initially and that there is a lower level of AFC occurring, meaning there is decreased total sodium transport. We found that the knockout value was increased over the wild-type for the wet:dry weight assay (Fig. 2A). For the Evan’s blue assay, a lower value indicates less absorption of fluid from the airspace (reduced AFC), also meaning there is reduced sodium transport in the lung. The value for knockout was significantly reduced compared with the wild-type for the Evan’s blue assay as well (Fig. 2B). *Both of these values indicate that overall sodium transport is reduced in the PKC-α knockout lung compared with wild-type.

Alveolar ENaC activity is decreased in PKC-α knockout mice. ENaC activity was recorded using cell-attached patch clamp on isolated ATII cells from PKC-α knockout mice and wild-type mice (Fig. 3A). The longer traces are representative single-channel records from wild-type or knockout ATII cells. ENaC activity is significantly decreased in the PKC-α knockout lungs vs. in the wild-type. The expanded regions of the traces show inward current with long mean open and close times, which are characteristic of ENaC (Fig. 3A). Figure 3B shows the current-voltage relationship for the channel in Fig. 3A. The inward rectification and highly positive reversal potential also distinguish ENaC. Figure 3C is a summary of the results from a large number of individual patches on ATII cells.
isolated from both wild-type and knockout animals. Wild-type data are from 29 individual patches; knockout data are from 32 isolated from both wild-type and knockout animals. Wild-type N

Asterisks indicate significant differences (P < 0.05) as determined using a t-test. A: the upper 2 traces are long representative records from knockout or wild-type cells (pipette potential is 40 mV). The activity of the knockout cell is substantially lower than that of the wild-type. The expanded regions are magnified 10-fold to emphasize the disparity in activity between the 2 groups. B: current-voltage relationship for the channel in A. The inward rectification and positive reversal potential are characteristic of ENaC. A summary of the single-channel data is shown in C–F. The graph in C shows that ENaC activity, measured as the product of the number of channels (N) times the open probability (Po), decreases more than 3-fold in the PKC-α knockout mice compared with wild-type (P < 0.002 by rank sum test). When the individual components of activity are examined, both Po (D) and N (E) decrease significantly in the knockout animals (for Po, P = 0.025; for N, P = 0.028, Mann-Whitney test). It is important when considering channel density that greater than 5-fold more empty patches were recorded from PKC-α knockout cells vs. wild-type (F, P < 0.001 by z-test). Numbers with the bars represent the number of individual patches made to determine the values for different parameters.

Knocking out PKC-α increases superoxide production in the alveoli. One trait that distinguishes alveolar ENaC from ENaC in other tissues is exposure to relatively high levels of oxygen and the concomitant elevated reactive oxygen species (ROS). High concentrations of ROS in the lung play an integral part in the signaling cascade involved in regulating lung fluid balance via ENaC (11). Using immunohistochemistry and confocal microscopy of lung slices, we examined superoxide production in the knockout mice compared with the wild-type mice. ATI cells are labeled green using green fluorescent protein-ECL, and superoxide is labeled with DHE, indicated by red color (as described in MATERIALS AND METHODS). Figure 4A, top, is divided into four images of the same slice. Figure 4A, top, left, shows the white light image merged with ECL and DHE; Figure 4A, top, right, shows ECL and DHE merged; Figure 4A, bottom, left shows DHE alone; Figure 4A, bottom, right, shows DHE merged with the white light image. Figure 4A, bottom, displays an enlarged view of the DHE fluorescence. The PKC-α knockout mice show higher levels of superoxide (Fig. 4B) than the wild-type mice (Fig. 4A). In data not shown, we used Image J to quantify the intensity of the DHE fluorescence over the entire field, and we observed a twofold increase in DHE intensity in the knockout animals, indicating that the superoxide levels are significantly elevated in the PKC-α knockout mice over the wild-type mice.

Knocking out PKC-α decreases SOD activity in the lung. PKC-α can decrease production of ROS via activation of SOD (27). The absence of PKC-α activation of SOD could explain the elevated ROS levels observed in the PKC-α knockout animals (Fig. 4B). Figure 4, D and E, summarizes the results of a SOD activity assay (as in MATERIALS AND METHODS) and

![Graphs and images](http://ajplung.physiology.org/)
Mitochondria | Cytosol

SOD activity measurements and fluorescent superoxide quantification strongly implicate an increase in ROS as the causative agent behind the change in ENaC activity in the PKC-α knockout lung.

ERK1/2 activity is increased in the PKC-α knockout lung. The ubiquitin ligase, Nedd4–2, controls ENaC channel density at the apical membrane (45, 46, 56). ERK1/2 phosphorylation of ENaC augments the ability of Nedd4–2 to ubiquitinate ENaC and promote internalization (4, 48). Increased ERK 1/2 activity would explain the decreased channel density of ENaC at the apical membrane observed in the PKC-α knockout mice (Figs. 1C and 3, E and F). We used ERK1/2 phosphorylation as a measure of ERK1/2 activity in the wild-type and knockout mice. We found that, although the total amount of ERK1/2 was comparable between the two groups, phosphorylated ERK1/2 was significantly increased in the knockout mice (Fig. 5), which likely explains the decreased ENaC channel density at the alveolar surface. It has been previously reported that ROS activates ERK1/2 (42). The increase in ERK1/2 activity in the

Fig. 4. Reactive oxygen species (ROS) levels are elevated in the PKC-α knockout lung. 125-μm lung slices were prepared from wild-type and PKC-α knockout mice. Subsequently, the slices were incubated in dihydroethidium (DHE), a fluorescent superoxide reporter, and Erythrina crista-galli lectin (ECL), an alveolar type I cell marker. The 2 panels are divided into 4 images: white-light image overlaid with DHE and ECL (top, left), DHE and ECL (top, right), DHE alone (bottom, left), and white-light image overlaid with DHE (bottom, right). White scale bar is 20 μm and is the same for all images. A: wild-type mice have lower levels of superoxide production than the knockout animals, as shown in B. DHE fluorescence was analyzed using confocal microscopy at excitation/emission 520/610 nm, and data were quantified using Image J. The intensity of the DHE fluorescence over the entire field was 2-fold greater in the knockout animals over the wild-type (P < 0.001 by t-test, n = 3 separate animals for each type). C and D: superoxide dismutase (SOD) activity is decreased in the PKC-α knockout lung. We measured both cytosolic and mitochondrial SOD activity using Superoxide Dismutase Assay Kit (Cayman Chemical). Samples were analyzed using a plate reader at excitation/emission 440/460 nm. Asterisks indicate significant differences as determined by t-test (P < 0.05). C: shows a significant decrease in cytosolic SOD activity in the knockout mice compared with wild-type (P = 0.034, 6 samples per mouse from 3 mice of each type). D: there is 2-fold decrease in mitochondrial SOD activity in the knockout lung vs. wild-type (P < 0.001; 6 samples per mouse from 3 mice of each type).
There was also a significant increase in the Po of wild-type animals where ROS levels were already minimal (Fig. 7D).

There was a significant increase in the channel density of ENaC at the membrane in the knockout mice after the addition of Tempol, whereas there was no significant change in the wild-type mice (Fig. 7C).

*Inhibition of MKP in wild-type cells mimics the reduction in channel density in knockout animals.* Phosphorylated ERK1/2 can phosphorylate ENaC and lead to ENaC internalization and degradation, thereby reducing the density of ENaC on the surface of ATII cells. The level of phosphorylation of ERK1/2 is determined by a balance between the rate at which ERK is phosphorylated by kinases, in particular PKC-δ, and the rate at which it is dephosphorylated by phosphatases, in particular MKP. Changes in activity of MKP can alter ENaC functional channel density (40), and increases in cytosolic ROS inhibit MKP (5, 31). Therefore, we investigated the effect of an MKP inhibitor on ENaC activity in wild-type and knockout cells. We predicted that inhibiting MKP in wild-type cells would increase ERK activity and make channel density in wild-type cells similar to that found in knockout cells. Also, because MKP should already be inhibited in knockout animals, there should be little effect of additional inhibition. Figure 8 shows that MKP inhibition does reduce channel density in wild-type cells but produces little effect on knockout cells. There appears to be little effect of MKP inhibitor on Po, which might be anticipated if the primary effect on Po is via MARCKS and not ERK.

*Alternate PKC isoforms present in the lung.* Whereas in renal principal cells there is only one detectable form of PKC, PKC-α (3), in alveoli there are multiple PKC isoforms (43); PKC-α, -β, -δ, -η, and -ζ can be detected in type II cells by immunoblotting (33). One of these isoforms, PKC-δ, is gener-

Fig. 5. Active ERK1/2 is increased in the PKC-α knockout lung. Using Western blotting, we measured total ERK1/2 and phosphoERK1/2 in whole lung homogenates from PKC-α knockout and wild-type mice (A). We used Image J to quantify the blots using an area that included both ERK1 and ERK2 bands. The program calculated the average density of the bands above the background for individual bands. B: the mean densitometry values from 3 separate experiments illustrating that total ERK is the same (P = 0.159), but phosphoERK is significantly increased in the knockout lung over the wild-type (*P = 0.020; n = 1 blot from 3 mice of each type).

**Channel density of ENaC increases with addition of Tempol.**

There was a significant increase in the channel density of ENaC at the membrane in the knockout mice after the addition of Tempol, but there was no significant change seen in the wild-type animals where ROS levels were already minimal (Fig. 7D). There was also a significant increase in the Po of ENaC in the PKC-α knockout mice after the addition of Tempol, whereas there was no significant change in the wild-type mice (Fig. 7C).

**MARCKS phosphorylation is increased in the PKC-α knockout lung.** One well-established mechanism for the regulation of ENaC Po involves the interaction of phosphatidylinositol phosphate (PIP2) with the channel (25, 35–37, 44, 54, 55). More recently, it has been shown that a specialized scaffolding protein, MARCKS, associates with PIP2, allowing for a high level of interaction with ENaC at the membrane. The phosphorylation state of MARCKS corresponds to its capacity to sequester PIP2 at the membrane in close proximity to ENaC; unphosphorylated MARCKS associates with the membrane and stabilizes PIP2 near ENaC, increasing Po, whereas phosphorylated MARCKS disassociates from the membrane and enters the cytosol, which lowers PIP2 concentrations in the vicinity of ENaC, causing ENaC Po to go down (1). We showed in Western blots that the levels of unphosphorylated MARCKS is comparable in the wild-type and knockout animals, but the levels of phosphorylated MARCKS is significantly increased in the PKC-α knockout mice, compared with the wild-type mice (Fig. 6). This increase in MARCKS phosphorylation explains the decrease in ENaC Po in the PKC-α knockout mice.

**Relative Band Density**

![Graph showing relative band density for ERK1/2 and pERK1/2](Image)

**Fig. 6.** Cytosolic MARCKS is increased in the PKC-α knockout lung. Using Western blotting, we measured total MARCKS and phosphoMARCKS in whole-lung homogenates from PKC-α knockout and wild-type mice (A). Unphosphorylated MARCKS protein is at the membrane, facilitating interaction that increase ENaC Po. When MARCKS is phosphorylated, it moves into the cytosol, causing a decrease in ENaC Po. We used Image J to quantify the blots. The program calculated the cumulative sum of the pixel values above the background for individual bands. Asterisks indicate significant differences as determined using a t-test (P < 0.05). B: mean densitometry values from 2 separate experiments, which show that total MARCKS is the same (P = n.s.), but phosphoMARCKS is significantly increased in the knockout lung over the wild-type (*P = 0.002; n = 1 blot from 3 mice of each type).
PKC-α KO Wild-type

the wild-type mice.

whereas there was no significant change in

P

knockout mice after the addition of Tempol

P

of Tempol (*

brane in the knockout mice after the addition

ENaC in wild-type cells.

A

: addition of Tempol has little effect on

activity was recorded from cell-attached

/H9251

wild-type mice or PKC-

patches on isolated alveolar type II cells from

PKC in wild-type cells. B: addition of Tempol to isolated alveolar type II cells from

PKC-α knockout mice resulted in a reversal

of the observed decrease in ENaC activity

compared with the wild-type. C: significant

increase in the P_o of ENaC in the PKC-α

knockout mice after the addition of Tempol

(*P < 0.001 by paired t-test, n = 14),

whereas there was no significant change in

the wild-type mice. D: significant increase in

the channel density of ENaC at the mem-

brane in the knockout mice after the addition

of Tempol (*P = 0.008), but there was no

significant change seen in the wild-type ani-

mals (n = 14).

ally inactive but is strongly activated by ROS (6). Because our

results show a role for ROS, we chose to investigate the levels

of PKC-δ in the PKC-α knockout vs. the wild-type lung. To

become active, the inactive form of PKC-δ must undergo

modification, including tyrosine phosphorylation (29) and pro-

teolytic cleavage. Using Western blotting, we observed that,

although there is no change in total levels of PKC-δ in the

PKC-α knockout lung compared with the wild-type lung, there

is a significant increase in the levels of active, phosphorylated,

and cleaved PKC-δ in the lungs of the PKC-α knockout mice

(Fig. 9).

Inhibition of PKC-δ increases ENaC activity in the PKC-α

knockout lung. If activation of PKC-δ is necessary for the

decrease in ENaC activity in the knockout mice, then inhibiting

PKC-δ should, at least partially, restore the activity. ENaC

activity was recorded from cell-attached patches on isolated

ATII cells (as in Fig. 3A), from wild-type mice or PKC-α

knockout mice, before and after addition of the PKC-δ inhibi-

tor. The addition of the PKC-δ inhibitor to isolated ATII cells

from the PKC-α knockout mice resulted in an increase of the

ENaC activity in the knockout cells (Fig. 10A) that makes the

activity comparable to that seen in wild-type (Fig. 10B). The P_o

of ENaC in the PKC-α knockout mice showed a significant

increase after the addition of the PKC-δ inhibitor, whereas in

the wild-type mice there was no significant change (Fig. 10C).

Concurrently, we observed a significant increase in the number

of ENaC channels at the apical membrane in the knockout mice

after the addition of the inhibitor, but there was no significant

change in the wild-type animals (Fig. 10D). This shows that the

inhibition of PKC-δ in the PKC-α knockout lung increases

ENaC activity by increasing P_o and by raising the density of

channels at the apical membrane.

Generalized activation and inhibition of PKC. Given the

information about the importance of both PKC-α and PKC-δ,

we could predict the effects of activation and inhibition of all

PKC isoforms in the lung. We would expect activation of PKC

with phorbol myristoyl acetate to produce a profound inhibi-

tion of ENaC P_o because both PKC-α and PKC-δ would

promote MARCKS phosphorylation and removal from the

membrane, which would subsequently lead to the loss of PIP2

activation of ENaC. General inhibition with an inhibitor that

blocks most isoforms of PKC (GF109203X) should, in con-

trast, produce dramatic activation of ENaC because, after

addition of the inhibitor, there will be no limitation on ENaC

P_o. Figure 11 shows that, in wild-type ATII cells, our predic-

tions are true.

DISCUSSION

The effect of PKC on ENaC in renal epithelial cells and in
distal nephron principal cells has been previously described in
the literature (3, 15, 32, 48, 53). With a few exceptions,
previous reports have generally shown that PKC activity in-
hibits ENaC. Interestingly, despite the role of PKC as a protein
kinase, ENaC does not appear to be directly phosphorylated
by PKC (53). Therefore, PKC must act indirectly to phosphorylate
one or more ENaC regulatory proteins. The purpose of this
work was to investigate which proteins might be modulated by
PKC to alter ENaC activity in the lung.

PKC isoforms in the lung. Alveolar epithelial cells contain
several different PKC isoforms. Transcripts encoding PKC-α,
-β, -δ, -ε, -η, and -ξ can be detected in type II cells by
RT-PCR, and PKC-α, -β, -δ, -η, and -ξ can be detected in type
II cells by immunoblotting (33). PKC-α knockout animals
presented us with an opportunity to determine the specific role
of one of these PKC isoforms, α, on ENaC-mediated alveolar
sodium transport.
work has shown that PKC-α downregulates both E-cadherin (50) and VE-cadherin (21), leading to an increase in barrier permeability following activation of PKC-α. This suggests a possibility of increased alveolar and capillary barrier function in PKC-α knockout mice. We demonstrated that there was an increase in the activity of other PKC isoforms in the PKC-α knockout animals, specifically an increase in PKC-δ activity. It has been reported that PKC-δ may have a barrier-protective function attributable to increasing focal adhesion points (22). The combination of the absence of a PKC-α-stimulated increase in endothelial permeability and a decrease in endothelial permeability caused by increased PKC-δ activation in the knockout animals suggests that the PKC-α knockout animals could have a less permeable endothelial barrier. This could also affect the observed alveolar fluid volume in the physiological measurements of AFC because the knockout animals with lower ENaC activity would have wetter lungs (which was observed), but, if there is decreased endothelial permeability, then the decreased permeability would partially compensate for the loss of ENaC function by reducing fluid movement into alveoli through paracellular pathways.

**AFC is reduced in the PKC-α knockout lung.** We used two separate methods, the wet-dry ratio and Evans blue, to show that fluid clearance in the lungs in PKC-α knockout mice was reduced. This suggests that the normal mechanisms for fluid uptake must be compromised. We subsequently showed that sodium uptake via ENaC was reduced. However, previous

**Fig. 8.** Inhibition of mitogen kinase phosphatase (MKP) in wild-type cells mimics the reduction in channel density in knockout animals. We examined the effect of the MKP1/3 inhibitor [((E)-2-Benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one] on ENaC activity in alveolar type II cells from wild-type and knockout animals. A and B: effect on channel density (measured as the number of measurable single channel levels in a patch). There is a significant reduction between untreated wild-type and MKP-inhibited wild-type (n = 4) and both knockout groups (indicated by an asterisk; n = 5). There is no significant difference between MKP-inhibited wild-type, untreated knockout, and MKP-inhibited knockout (determined by 1-way ANOVA). C and D: there appears to be no effect of MKP inhibition on P_o in either wild-type or knockout cells (paired t-test, n = 4 and 5, respectively).

**Fig. 9.** Active PKC-δ is increased in the PKC-α knockout lung. Using Western blotting, we measured total PKC-δ in whole lung homogenates from PKC-α knockout and wild-type mice. To become active, the inactive form of PKC-δ must undergo modification. A: blot from alveolar type II cell lysates from wild-type and knockout animals (above). We determined that the amount of active PKC-δ, represented by the bottom band with a molecular weight around 50 kDa, is increased compared with the inactive form (*P < 0.01, n = 6). B: we repeated the experiment in the presence of instilled PKC-δ inhibitor to show that the inhibitor reduces the active form of PKC-δ (n = 6, difference in the ratio is not significant). We used Image J to quantify the blots. The program calculated the cumulative sum of the pixel values above the background for individual bands.
PKC-α (3, 43). Thus, in principle, cells knocking out PKC-α eliminates all PKC-mediated phosphorylation. In contrast, in alveolar epithelial cells, alternative isoforms can promote PKC phosphorylation. In particular, one isoform, PKC-δ, is activated by ROS (6, 38). The mechanism by which P0, is increased likely involves PIP2 interaction with the channel. That ENaC gating depends on PIP2 has been well known for a long time (25, 35–37, 44, 54). Recently, however, Alli et al. (1) have shown that the local concentration of PIP2 in the membrane is controlled by association with the apical membrane of a specialized protein, MARCKS (or the very similar MARCKS-related protein). The ability of MARCKS to control the local concentration of PIP2 in the membrane near ENaC depends on the state of MARCKS phosphorylation; when dephosphorylated, MARCKS associates with the membrane, and membrane PIP2 concentrations are elevated, which leads to increased ENaC P0, when phosphorylated, MARCKS leaves the membrane and enters the cytosol, which reduces PIP2 concentrations near ENaC, which decreases ENaC activity. The primary kinase that phosphorylates MARCKS in alveolar epithelial cells is PKC-δ (41). Thus, when PKC-δ is activated by ROS, MARCKS is phosphorylated and leaves the membrane-decreasing PIP2 concentrations near ENaC and reduces ENaC P0 (as we observed).

**ENaC apical membrane density is decreased in the PKC-α knockout lung.** In addition to a decrease in P0, we also observed an unexpected decrease in ENaC channel density at the apical membrane of the alveoli in the knockout lung. One known mechanism for reducing ENaC membrane density is via phosphorylation by active ERK1/2, which promotes ENaC interaction with the ubiquitin ligase Nedd4–2. In turn, ubiquitination by Nedd4–2 promotes ENaC internalization, therefore reducing the apical channel density at the membrane (4, 46, 52, 56). ROS-activated PKC-δ can phosphorylate and activate ERK1/2 (16–18). Also, ROS inhibits MKP, which leads to decreased ERK1/2 dephosphorylation (2, 42, 47). The combination of
PKC-δ activation and MKP inhibition leads to elevated ERK phosphorylation and ERK1/2-dependent ENaC internalization.

Superoxide levels and ROS, in general, play a key role in regulating alveolar ENaC and the maintenance of normal lung function (11, 11, 24). Previous literature has consistently reported that ROS has a stimulatory effect on ENaC activity (2, 9–11, 19, 24, 38, 47, 51). We were surprised to find that, in certain circumstances, elevated ROS can cause a decrease in ENaC activity, as seen in the PKC-α knockout lung. It seems that there are different roles for ROS in the regulation of ENaC, both inhibitory and stimulatory. We hypothesize that one unique characteristic of ROS signaling in the lung, which could lead to opposing regulatory effects on ENaC, is whether the ROS originated intracellularly or extracellularly. The absence of PKC-α in the lung affects intracellular ROS, whereas most of the previous literature, in both the kidney and the lung, in vivo and ex vivo, was concerned with the effect of extracellular ROS on ENaC activity. We will be interested to investigate in the future the discrete effects of extracellular and intracellular ROS on the regulation of alveolar ENaC.

Figure 12 shows a schematic diagram of PKC signaling in the wild-type and knockout lung. Figure 12A shows the wild-type lung. PKC-α is active, stimulating SOD, which breaks down ROS. In the absence of ROS, PKC-δ is inactive and does not phosphorylate MARCKS protein, which allows MARCKS to sequester PIP2 near ENaC in the membrane, increasing ENaC Po. In addition, ERK1/2 is not phosphorylated by PKC-δ, so that ENaC density at the membrane is increased because of decreased internalization. In addition, without high levels of superoxide in the lung, MKP is active, which dephosphorylates ERK1/2, leading to reduced apical expression of ENaC. Figure 12B shows that, in the knockout lung, in the absence of PKC-α, SOD is less active, leading to elevated ROS. ROS activates PKC-δ and inhibits MKP. The combination leads to increased ERK1/2 phosphorylation via PKC-δ and decreased dephosphorylation via MKP, which together activate ERK. ERK in turn phosphorylates ENaC, which promotes interaction with Nedd4-2, causing the ubiquitination and subsequent internalization of ENaC. ROS activation of PKC-δ promotes phosphorylation of MARCKS protein, which, when phosphorylated, leaves the membrane and does not sequester PIP2 in proximity to ENaC. Without this interaction with PIP2, ENaC Po is decreased.

Our findings may also shed light on our understanding of AFC under basal vs. stimulated conditions. Although our experiments were conducted under basal conditions, the increase in ROS seen in the knockout animals partially mimics stimulated conditions in the alveoli. It has been previously reported that, under stimulated conditions, such as during infection by virus or treatment with LPS, a PKC inhibitor reversed downregulation of ENaC activity (20, 24, 31a). Lazrak, et al. (31a) also demonstrated that the mechanism of decreased ENaC activity was due to increased ROS levels. Similarly, LPS is known to increase ROS levels and caused a similar decrease in ENaC activity (20). Figure 12 illustrates this idea. Under basal conditions in WT mice, only low levels of PKC-α are active, and no other PKC isoform is active. Application of phorbol ester initially activates PKC-α, which

**Fig. 12.** Schematic diagram of alveolar PKC signaling in wild-type and PKC-α knockout mice. A: wild-type lung. PKC-α is active, stimulating SOD, which breaks down ROS. In the absence of ROS, PKC-δ is inactive and does not phosphorylate MARCKS protein, which allows MARCKS to sequester phosphatidylinositol phosphate (PIP2) near ENaC in the membrane, increasing ENaC Po. In addition, ERK1/2 is not phosphorylated by PKC-δ, so that ENaC density at the membrane is increased because of decreased internalization. In addition, without high levels of superoxide in the lung, MKP is active, which dephosphorylates ERK1/2, leading to reduced apical expression of ENaC. B: in the knockout lung, in the absence of PKC-α, SOD is less active, leading to elevated ROS. ROS activates PKC-δ and inhibits MKP. The combination leads to increased ERK1/2 phosphorylation via PKC-δ and decreased dephosphorylation via MKP, which together activate ERK. ERK in turn phosphorylates ENaC, which promotes interaction with Nedd4-2, causing the ubiquitination and subsequent internalization of ENaC. ROS activation of PKC-δ promotes phosphorylation of MARCKS protein, which, when phosphorylated, leaves the membrane and does not sequester PIP2 in proximity to ENaC. Without this interaction with PIP2, ENaC Po is decreased.
reduces ENaC Po. This effect can be blocked with a PKC-α inhibitor (GÖ6976). Continued exposure to phorbol ester increases ROS, stimulates PKC-δ, and downregulates PKC-α. Downregulation of PKC-α leads to downregulation of ENaC translation (48). In the knockout mice, absence of PKC-α downregulates PKC-α inhibitor (GÖ6976). Continued exposure to phorbol ester inhibits PKC-δ, and downregulates PKC-α.

### DISCUSSIONS

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

### REFERENCES


