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Local small airway epithelial injury induces global smooth muscle contraction and airway constriction

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Zhou J, Alvarez-Elizondo MB, Botvinick E, George SC. Local small airway epithelial injury induces global smooth muscle contraction and airway constriction. *J Appl Physiol* 112: 627–637, 2012. First published November 23, 2011; doi:10.1152/japplphysiol.00739.2011.—Small airway epithelial cells form a continuous sheet lining the conducting airways, which serves many functions including a physical barrier to protect the underlying tissue. In asthma, injury to epithelial cells can occur during bronchoconstriction, which may exacerbate airway hyperreactivity. To investigate the role of epithelial cell rupture in airway constriction, laser ablation was used to precisely rupture individual airway epithelial cells of small airways (<300-μm diameter) in rat lung slices (~250-μm thick). Laser ablation of single epithelial cells using a femtosecond laser reproducibly induced airway contraction to ~70% of the original cross-sectional area within several seconds, and the contraction lasted for up to 40 s. The airway constriction could be mimicked by mechanical rupture of a single epithelial cell using a sharp glass micropipette but not with a blunt glass pipette. These results suggest that soluble mediators released from the wounded epithelial cell induce global airway contraction. To confirm this hypothesis, the lysate of primary human small airway epithelial cells stimulated a similar airway contraction. Laser ablation of single epithelial cells triggered a single instantaneous Ca^{2+} wave in the epithelium, and multiple Ca^{2+} waves in smooth muscle cells, which were delayed by several seconds. Removal of extracellular Ca^{2+} or decreasing intracellular Ca^{2+} both blocked laser-induced airway contraction. We conclude that local epithelial cell rupture induces rapid and global airway constriction through release of soluble mediators and subsequent Ca^{2+}-dependent smooth muscle shortening.

calcium; epithelial cells; laser ablation; soluble mediators

THE AIRWAY EPITHELIUM IS A continuous sheet of cells lining the conducting airways, which serves many functions including a physical barrier to protect the underlying tissue from inflammatory and physical stimuli. As such, the airway epithelium is susceptible to injury following exposure to harmful chemical stimuli such as environment pollutants (56), toxic gases (21, 59), or viruses (41, 73–74), and physical stimuli such as mechanical compression during bronchoconstriction (90) or surface tension created by moving air bubbles in a fluid-filled lung (44). In response to injury, airway epithelial cells initiate a rapid wound healing process through activation of basal epidermal growth factor receptor (86) and increase the secretion of soluble mediators such as transforming growth factor (TGF)-β_1 and -β_2, interleukin (IL)-6, IL-8, and IL-13, matrix metalloproteinase (MMP)-9, and platelet-derived growth factor (PDGF) to increase smooth muscle proliferation and tissue remodeling (55, 62, 68, 80, 94). Mechanical stimulation or wounding of single epithelial cells has been shown to induce Ca^{2+} waves in both epithelial monolayer culture (14, 34, 37, 69, 93) and the intact tracheal epithelium (25). However, it is not known whether disruption of the epithelial cell itself can lead directly to airway constriction.

Inflammation and bronchoconstriction in the small airways in asthma are major sources of morbidity (33, 36, 40, 48, 85). Thus we investigated the role of single epithelial cell rupture on airway constriction in small airways (<300-μm diameter) by combining an ex vivo lung slice model with a laser ablation technique. The tissue slice model has been widely used to study pulmonary physiology including smooth muscle cell contractility and airway wall mechanics (6, 12, 46, 53, 57–58, 66, 70–71, 91–92). In the lung slices, small airways maintain near normal anatomical dimensions between epithelial cells, smooth muscle cells, and the extracellular matrix. Furthermore, by inflating the lungs with agarose gel before obtaining the slices, the airways maintain similar tissue mechanical properties as in vivo air-filled lungs (2). Pulsed nanosecond or femtosecond laser has been used extensively to disrupt subcellular structures such as single stress fibers (50), microtubules (15), and mitochondria (72, 89) or cellular structures such as the cell membrane (82). The technique was adopted in this study to precisely rupture single epithelial cells in the three-dimensional airways of the lung slices. In contrast to other methods used to damage epithelial cells, such as mechanical compression or scrape using glass needles or pipette tips, laser ablation is more precise and rapid.

We hypothesized that epithelial cell injury could lead to airway constriction. To address this hypothesis, we used a pulsed femtosecond laser to ablate epithelial cells in small airways. Our results demonstrate that laser ablation of single epithelial cells induces airway contraction within seconds by triggering different patterns of Ca^{2+} waves in epithelial and smooth muscle cells. The laser-induced airway contraction was dependent on smooth muscle extracellular and intracellular Ca^{2+} but did not depend on the epithelial Ca^{2+} wave. We demonstrate for the first time that local epithelial injury induces rapid (within seconds) and global airway constriction through biochemical communication between the wounded epithelial cell and surrounding smooth muscle cells.

MATERIALS AND METHODS

Materials. Fluo-4/AM, CM-H2DCFDA, Pluronic F-127, HBSS, DMEM, antibiotic-antimycotic, and the live/dead assay were purchased from Invitrogen (Carlsbad, CA). Sulfobromophthalein, *N*-acetylcysteine, propidium iodide, thapsigargin, caffeine, suramin, acetyl-

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choline, nifedipine, ATP, and atropine were purchased from Sigma-Aldrich (St. Louis, MO). Pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid tetrasodium salt (PPADS) and ryanodine were purchased from Tocris Bioscience (Ellisville, MO). Diinosine pentaphosphate (Ip5I) was purchased from TimTec (Newark, DE). Supplemented HBSS (sHBSS) was made from HBSS with Ca^{2+} and Mg^{2+} supplemented with 20 mM HEPES buffer (pH 7.4) and then sterilized using a 0.2-μm filter (12). Ca^{2+}-free HBSS was made from HBSS without Ca^{2+} and Mg^{2+} supplemented with 20 mM HEPES buffer, 0.4 mM $MgSO_4$, 0.5 mM $MgCl_2$, and 4 mM EGTA.

Preparation of lung tissue slices. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine, and were consistent with National Institutes of Health guidelines. Lung tissue was collected from 2- to 4-wk-old Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA). Rats (30–50 g) were deeply anesthetized with isofurane and decapitated. To solidify lung tissue, 1.75% agarose (low melting/gelling temperature, type VII-A; Sigma-Aldrich) was inflated into the lungs. Briefly, low gelling temperature agarose was dissolved in distilled water at 65°C, mixed with sHBSS, and then cooled to 37°C. A blunt 20-gauge steel needle was inserted into the rat trachea and clamped with a hemostat (Fine Science Tools, Foster City, CA). Approximately 50 ml/kg of the 37°C agarose solution, which reaches the total lung capacity of Sprague-Dawley rats (49), was slowly added to the lungs through the trachea, and then ~0.3 ml of air was injected to empty the agarose from the lumens of the airways. The animal was then covered with ice for 3–5 min to lower the temperature and solidify the agarose solution. The whole lungs were then rapidly removed and kept in ice-cold sHBSS for 20 min. The lung tissue was cut into 250-μm thick slices using an oscillating tissue slicer (EMS-4000; Electron Microscopy Sciences, Fort Washington, PA). The slices were transferred to DMEM (Invitrogen) supplemented with antibiotics and antimycotics in a 37°C, 5% CO_2 incubator.

Measurement of intracellular Ca^{2+} signaling. To monitor free intracellular Ca^{2+} in both epithelial and smooth muscle cells, lung tissue slices were incubated in 2 ml of sHBSS with 20 μM Fluo-4/AM (Invitrogen), 100 μM sulfobromophthalein (Sigma-Aldrich), and 0.2% Pluronic F-127 (Invitrogen) for 1 h at room temperature (6). Subsequently, the slices were kept in sHBSS with 100 μM sulfobromophthalein for another hour at room temperature. The slices were then transferred to a glass bottom dish (MatTek, Ashland, MA) and held in place with a slice anchor (Warner Instruments, Hamden, CT). Confocal imaging was performed on a Zeiss 510 Meta multiphoton laser scanning microscope (LSM 510; Zeiss, Jena, Germany). The speed of Ca^{2+} imaging was 1.57 s/frame (2.56 μs/pixel). Briefly, Fluo-4 was excited with a 488-nm laser, and the fluorescence images (512 × 512 pixels) were collected. Regions of interest (ROI; 3 × 3 pixels) were selected in individual smooth muscle cells using ImageJ (v. 1.42; Wayne Rasband, National Institutes of Health) to track the changes in fluorescence intensity. We manually changed the position of the ROI to track the Ca^{2+} signal in the same cells when they were contracting. The ratio of fluorescence intensity (F/F_0) was calculated by dividing fluorescence intensity at time t (F) with the intensity at the beginning of the experiment (F_0).

Laser ablation. The femtosecond (fs) laser ablation was performed on the LSM 510 with an Achroplan 40×/0.8 NA water-immersion objective. A single epithelial cell, located 30–75 μm from the bottom of the slice in z-direction (Fig. 1*A*), was ruptured by focusing the Mode-locked Ti:Sapphire femtosecond laser beam over a triangular ROI (~6 μm^2) that covered the apical membrane of the epithelial cell. The ROI was scanned horizontally by the femtosecond laser at 100 μs/μm. By using the "bleach control" program in the LSM510, we were able to immediately (<1 s) switch between the imaging mode and the ablation mode. The femtosecond laser beam was produced from a Coherent Chameleon system (Coherent, Santa Clara, CA) with 800-nm wavelength, 140-fs pulse duration, and 80-MHz repetition rate. The average power before the objective was ~800 mW, which was measured with a laser power and energy meter (Fieldmaster GS; Coherent). The average power at the sample plane was ~600 mW considering an objective transmission efficiency of 75%. The pulse energy for our 80-MHz laser was ~7.5 nJ per pulse (600 mW/80 MHz), and the peak power was 37.5 kW.

Preparation of epithelial cell lysate. Primary human small airway epithelial cells (SAECs) were purchased from Lonza (Walkersville, MD) and grown on 150 × 15 mm tissue culture dish (Sarstedt, Newton, NC) in small airway epithelial basal medium supplemented with growth factors supplied in the SAGM SingleQuot kit (Lonza) in a 37°C, 5% CO_2 incubator. SAEC lysate was freshly made before each experiment. Since fetal bovine serum (FBS) has been shown to induce airway smooth muscle contraction (1), care was taken to remove FBS in the medium before lysing the cells. Briefly, basal medium was aspirated from the tissue culture dish and the SAECs were washed three times with sHBSS to remove any residual FBS. Four milliliters of fresh sHBSS were then added to the tissue culture dish, and 1 ml sHBSS was taken as SAEC debris control solution. To attain SAEC debris, SAECs were first physically scraped from the tissue culture dish using a sterile tissue scraper (Sarstedt, Newton, NC). The remaining 3 ml sHBSS containing SAECs were then centrifuged, 2 ml supernatant were removed, and the cell pellet was extensively agitated to disrupt the cell membranes. The final cell density in the epithelial cell debris, which is used to induce airway contraction, was ~3,600 cells/ml.

Mechanical stimulation and wounding of epithelial cells in small airways. Sharp glass micropipettes (~1 μm tip diameter) mounted on a three-dimensional manual micro-manipulator (Newport, Irvine, CA) were used to mechanically break the membrane of epithelial cells in small airways. Briefly, a sharp glass micropipette filled with sHBSS was applied with positive pressure via a 3-cc plastic syringe. The tip of the micropipette was lowered to penetrate into epithelium, and then the micropipette was manually pulled forth and back to break the epithelial cell membrane. Blunt glass pipettes were prepared by fire polishing the tip of sharp glass micropipettes to make a rounded closed end, which prevents the epithelial cell injury during mechanical stimulation due to the large contact area between the round end and the epithelium. The epithelium was mechanically stretched by manually pushing and pulling the blunt glass pipette using the micromanipulator or moving the stage of the microscope. As a positive control, laser ablation was performed to induce airway contraction after each mechanical stimulation experiment.

Data analysis. The ratio of lumen area was defined as the minimum cross-sectional area of airways after treatment divided by initial cross-sectional area. Statistical tests of significance of the ratio of lumen area were carried out with the nonparametric Mann-Whitney U-test using commercial software (SPSS v. 16; Chicago, IL), and $P < 0.05$ was considered statistically significant.

RESULTS

Laser ablation of single epithelial cells induced airway contraction. To investigate the role of epithelial cell rupture in airway constriction, a pulsed femtosecond laser was targeted at the apical surface of single epithelial cells (Fig. 1*A*). The pulsed laser disrupted the membrane of a single epithelial cell and induced global airway contraction (Fig. 1*B* and Supplemental Movie S1; Supplemental Material for this article is available online at the *J Appl Physiol* website). By measuring the lumen area at different time points following laser ablation, we found that damage of single epithelial cells induced airway contraction within seconds. The contraction was steady leading to a maximum in ~40 s in which the airway lumen was ~70% of the original cross-sectional area (Fig. 1, *C* and *D*). We found that the airway contraction induced by laser ablation recovered ~90% of the preceding lumen area after several minutes (Fig.

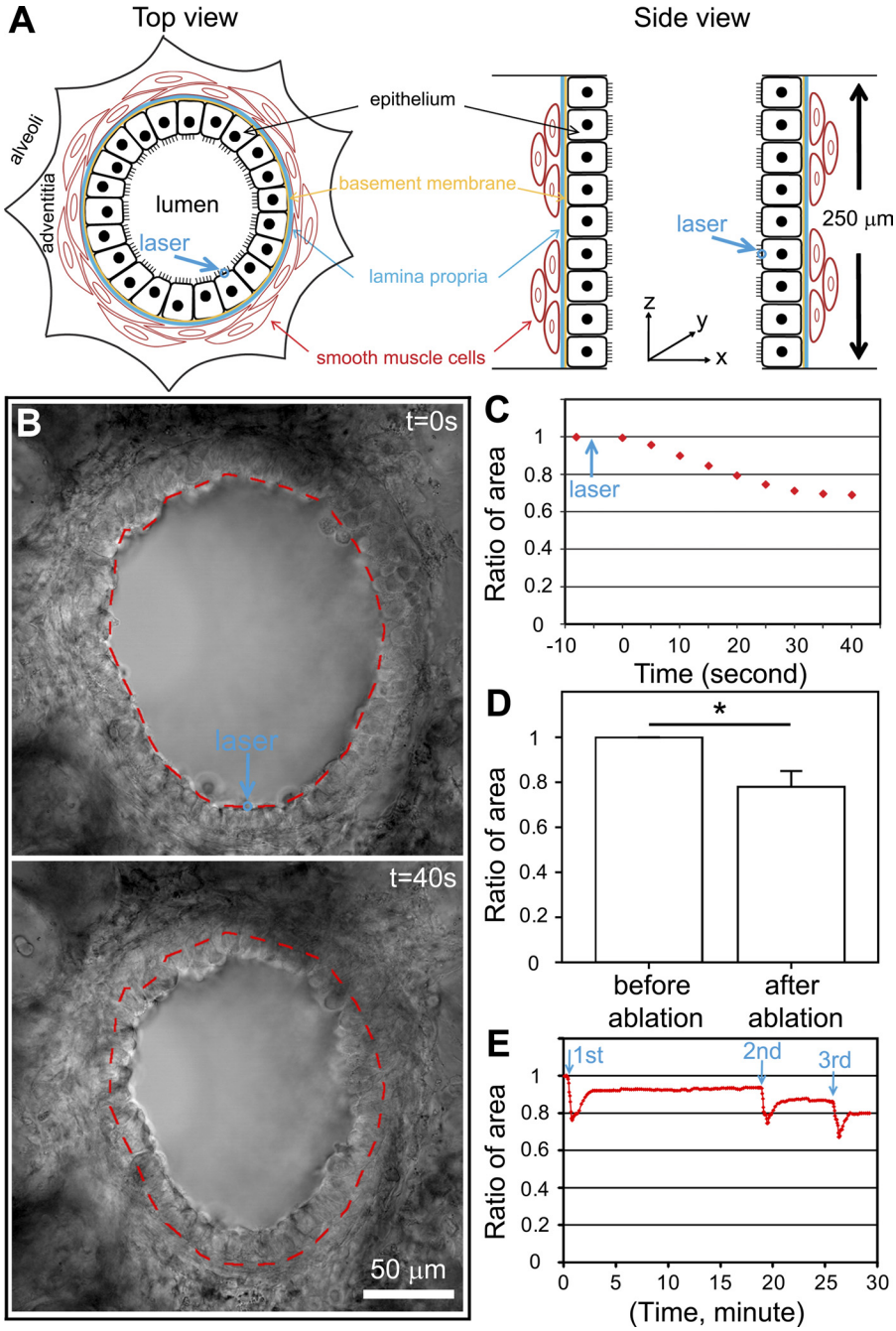

Fig. 1. Laser ablation of single epithelial cells induced airway contraction. *A*: schematic representation of the structure of a small airway (*left*, top view; *right*, side view) in a lung tissue slice. Pulsed femtosecond laser was targeted at the apical surface of a single epithelial cell, which is located 30–75 μm from the bottom of the slice in *z*-direction. *B*: pulsed laser disrupted the epithelial cell membrane and induced global airway contraction. Blue arrows point to the ablated epithelial cell. Red dashed line outlines the lumen cross-sectional area before laser ablation. *C*: damage of single epithelial cells induced airway contraction within seconds. Contraction was steady leading to a maximum in ~40 s in which the airway lumen was ~70% of the original cross-sectional area. *D*: statistical tests demonstrate that the ratio of lumen area was significantly different before and after laser ablation ($n = 7$ airways from 5 different rats). *E*: airway contracts in response to the multiple single epithelial cell ablations in succession. Lumen area recovers to ~90% of the starting area after several minutes following the first, second, and third successive laser ablation of neighboring cells.

1*E*) and the response was repeatable when single epithelial cells were ablated in succession following the recovery phase (Fig. 1*E* and Supplemental Movie S2).

Soluble mediators released from the wounded epithelial cell induced airway contraction. In our studies, we adopted the pulsed laser to ablate epithelial cell membranes by the plasma-induced ablation of the tissue. However, the laser can affect biological tissue through other effects including photochemical, photothermal, and photomechanical processes such as stress wave emission and cavitation bubble dynamics (17, 87–88). These alternative effects could directly stimulate or damage other neighboring cells such as the underlying smooth muscle cells. The following experiments were performed to further characterize the laser-mediated effects of epithelial cell ablation.

It has been shown that a pulsed laser increases the production of reactive oxygen species (ROS; Refs. 9, 81), which could induce smooth muscle contraction (47, 54, 79). To investigate the role of ROS in laser-induced airway contraction, we used CM-H2DCFDA, a ROS-sensitive fluorescence indicator, to monitor the level of ROS (24). We did not detect any increase in ROS activity after laser ablation. Furthermore, a ROS inhibitor, *N*-acetylcysteine (54), did not block the laser-induced airway contraction (data not shown).

A high energy pulsed laser can transiently open cell membranes via stress wave propagation (82). To determine the damage zone

induced by the pulsed laser in our experiments, 20 ug/ml propidium iodide were added to the bath medium to monitor the membrane integrity and cell viability of both epithelial and smooth muscle cells. We found that the pulsed femtosecond laser only damaged the apical membrane of the ablated epithelial cell and had no effects on neighboring epithelial cells or underlying smooth muscle cells in the *x*, *y*, and *z* direction (Fig. 2*A* and Supplemental Movie S3 and S4), indicating that the effects of laser ablation was local.

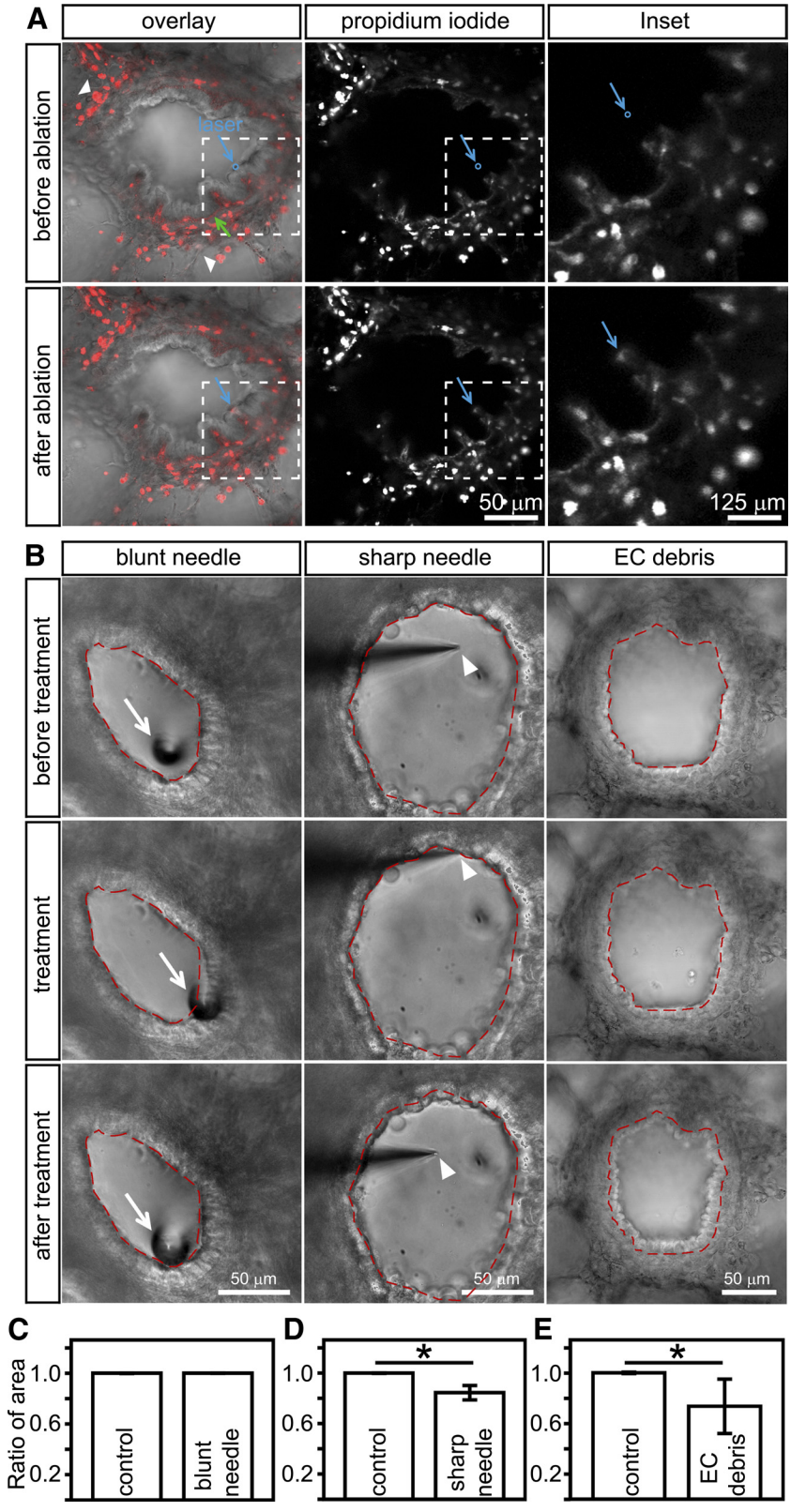

Fig. 2. Soluble mediators released from the wounded epithelial cell induced airway contraction. *A*: lung slices were stained with propidium iodide and demonstrate that a pulsed femtosecond laser only damaged the ablated epithelial cell (EC) but had no effects on neighboring epithelial cells or underlying smooth muscle cells. White dashed line outlines the ablated epithelial cell and defines the area being magnified in the third column. Blue arrows point to the ablated epithelial cell. Green arrows point to the extracellular matrix. White arrowheads point to the dead epithelial and smooth muscle cells. *B*: mechanical stimulation of airway wall with a blunt glass needle did not induce airway contraction, in contrast to mechanically injuring epithelial cells with a sharp glass needle or applying epithelial cell debris. White arrows and arrowheads point to the tip of the blunt glass needle and sharp glass needle, respectively. Red dashed line outlines the lumen cross-sectional area before laser ablation. *C*: statistical tests demonstrate that there was no significant difference in the ratio of lumen area between control and blunt needle stimulated groups. *D*: mechanical wounding of epithelial cells significantly reduced airway cross-sectional area. *E*: epithelial cell debris significantly decreased airway cross-sectional area ($n = 4–6$ airways from a minimum of 2 different rats for each condition). *$P < 0.05$.

Mechanical stretch of airway smooth muscle, which could be induced by the laser-mediated stress wave propagation or moving cavitation bubbles during laser ablation, has been shown to increase the contractility of smooth muscle cells (38–39). To test the role of mechanical stretch of epithelial and smooth muscle cells in airway contraction, we used a blunt glass pipette to mechanically deform the airway wall. The blunt glass pipette has a large contact surface with the epithelium so that it prevents damage to the epithelial cell membrane during mechanical stimulation. The blunt glass pipette stretched both epithelial cells and underlying smooth muscle cell layer but did not change the lumen area (Fig. 2, *B* and *C*, and Supplemental Movie S5).

To test the role of epithelial injury in airway contraction, we applied a sharp glass micropipette (~1-μm tip diameter) to mechanically rupture the epithelial cell membrane. Since the cilia of airway epithelial cells beat frequently when the cells are alive and stop beating when dead, the beating cilia were used to indicate cell viability before sharp-needle stimulation, and the cessation of beating cilia after stimulation was used to demonstrate successful damage of the targeted cell. Using a sharp glass micropipette, we were able to mechanically wound individual epithelial cells and induce airway contraction (Fig. 2, *B* and *D*, and Supplemental Movie S6).

Since mechanical wounding of epithelial cells induced airway contraction, we hypothesized that soluble mediators released from the wounded epithelial cell stimulate airway contraction. To confirm this hypothesis, we added the lysate of primary human SAECs to the bath medium and found that SAEC debris induced a similar airway contraction (Fig. 2, *B* and *E*, and Supplemental Movie 7). Control solution for SAEC debris (sHBSS) had no effect on the airway lumen area (Fig. 2*E*).

Ca^{2+} waves in epithelial and smooth muscle cells. The laser-induced airway contraction could be directly mediated by contraction of the epithelial sheet as in embryonic tissue (26, 43, 75) or could be mediated by smooth muscle cell contraction indirectly induced by soluble mediators released from the wounded epithelial cell. We found that laser ablation of a single epithelial cell triggered Ca^{2+} waves in both the epithelium and smooth muscle layer; however, the patterns of the Ca^{2+} waves were different. In epithelial cells, the Ca^{2+} wave started immediately from the ablated cell, spread to neighboring epithelial cells in both directions, and lasted only one cycle (Fig. 3, *A–C*, and Supplemental Movie S8). In contrast, the initial Ca^{2+} wave in smooth muscle cells was delayed by several seconds and continued for multiple cycles (Fig. 3, *B* and *C*). Airway contraction corresponded to Ca^{2+} oscillations in the smooth muscle cells (Fig. 3*D*).

To investigate the role of extracellular Ca^{2+} in laser-induced airway contraction, we incubated lung tissue slices in Ca^{2+} free HBSS with 4 mM EGTA for 5 min. In the absence of extracellular Ca^{2+}, laser ablation of single epithelial cells induced the Ca^{2+} wave in the epithelium (Fig. 4*A* and Supplemental Movie S9) but did not trigger Ca^{2+} oscillations in smooth muscle cells and did not induce airway contraction (Fig. 4, *A* and *B*). These results suggest that Ca^{2+} oscillations in smooth muscle cells might be dependent on Ca^{2+} influx. To confirm this, we found that 10 μM nifedipine, an L-type voltage-dependent Ca^{2+} channel inhibitor, blocked Ca^{2+} oscillations in smooth muscle cells and airway contraction induced by epithelial injury (Fig. 4*B*) but did not block the Ca^{2+} wave in epithelial cells (data not shown).

The Ca^{2+} influx might stimulate internal Ca^{2+} release through ryanodine receptors, a mechanism called calcium-induced calcium release. To test this mechanism, we blocked the increase in intracellular Ca^{2+} using a 45-min treatment of 50 μM ryanodine, which maintains the ryanodine receptor and coupled Ca^{2+} release channels in a close state (61). We found that ryanodine treatment blocked Ca^{2+} oscillations in smooth muscle cells and airway contraction induced by epithelial injury (Fig. 4*C*) but did not block the Ca^{2+} wave in epithelial cells (data not shown). To confirm this, we treated the tissue with a combination of ryanodine (50 μM) and caffeine (20 mM) to lock the ryanodine receptor and coupled Ca^{2+} release channels in an open state and thus clamp the intracellular Ca^{2+} (6). After 5 min, ryanodine and caffeine were washed out, but the intracellular Ca^{2+} was clamped at a constant level and was controlled by changing extracellular Ca^{2+} for the next 1–2 h. When intracellular Ca^{2+} was clamped at a normal Ca^{2+} level, the rat airway contracted very slowly (Supplemental Movie S10*A*). We found that clamping the intracellular Ca^{2+} at a normal Ca^{2+} level (Fig. 4*C* and Supplemental Movie 10*B*) or at zero Ca^{2+} level (data not shown) blocked the Ca^{2+} oscillations in smooth muscle cells and the rapid airway contraction induced by single epithelial cell injury.

To further confirm the role of intracellular Ca^{2+}, we blocked the increase in intracellular Ca^{2+} by 30-min treatment of 10 μM thapsigargin, an inhibitor of the sarco(endo)plasmic reticulum Ca^{2+}-ATPases, which empties the Ca^{2+} store to prevent the changes in intracellular Ca^{2+}. When intracellular Ca^{2+} was emptied, the rat airway contracted very slowly and to a lesser degree (Supplemental Movie 11*A*). We found that inhibition of intracellular Ca^{2+} significantly blocked the Ca^{2+} oscillations in smooth muscle cells and the rapid airway contraction induced by laser ablation of single epithelial cells (Fig. 4*C* and Supplemental Movie 11*B*).

It has been demonstrated that Ca^{2+} intercellular communication between epithelial cells is mediated by the intercellular diffusion of IP_3 via gap junctions or release and subsequent diffusion of ATP through the extracellular space to neighboring epithelial cells in response to mechanical stimulation or wounding (14, 18–19, 28, 34, 37, 42, 69). To study the role of ATP in mediating the Ca^{2+} communication between epithelial and smooth muscle cells, we used suramin, a nonselective P2 purinergic antagonist to block the ATP receptors on epithelial and smooth muscle cells. We found that 300 μM suramin blocked the Ca^{2+} wave in epithelium (Fig. 5*A* and Supplemental Movie S12) but did not inhibit the Ca^{2+} oscillations in smooth muscle cells and airway contraction induced by laser ablation of single epithelial cells (Fig. 5*B* and Supplemental Movie S12). To confirm the results, we found that apyrase and PPADS, which can enzymatically remove ATP and block the P2X receptors, respectively, did not inhibit the airway contraction induced by epithelial cell injury (data not shown). Since it has been suggested that ATP-induced rat airway smooth muscle contraction can occur through P2X receptors (63) and suramin might not be able to inhibit some P2X receptors such as P2X1 and P2X3 (31), we applied Ip_5I, a P2X1 and P2X3 antagonist (31), and found that Ip_5I did not block the airway contraction induced by epithelial injury (Fig. 5*B*). However, we found that ATP induced contraction

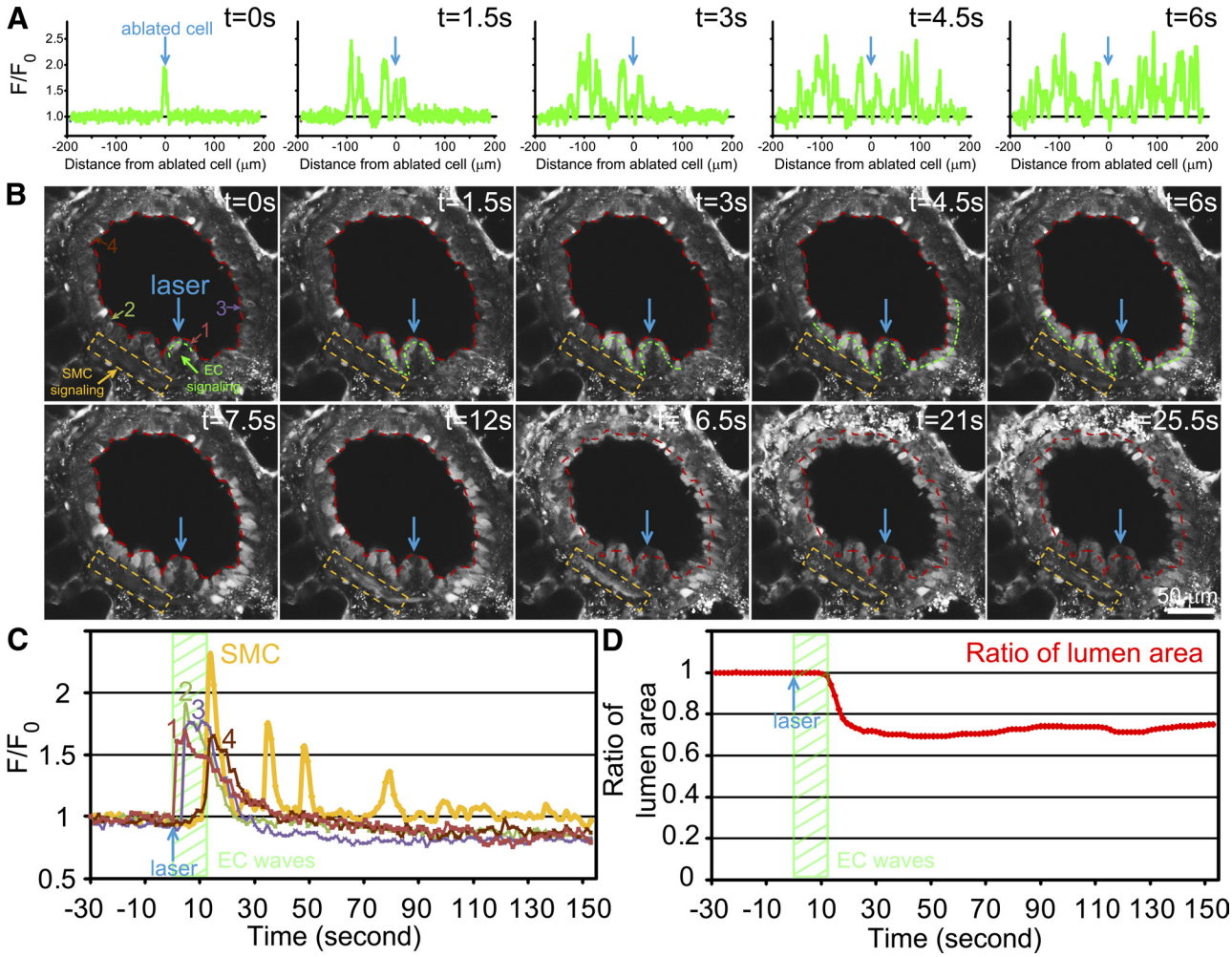

Fig. 3. Ca^{2+} waves in epithelial and smooth muscle cells (SMC). *A*: Ca^{2+} signaling in the ablated epithelial cell immediately increased and rapidly (seconds) spread to neighboring epithelial cells. *B*: laser ablation of a single epithelial cell triggered a series of events. In the first ~10 s, the Ca^{2+} wave spread from ablated cells to neighboring epithelial cells in both directions, but there was no increase of Ca^{2+} signaling in smooth muscle cells. Then, Ca^{2+} oscillations began in smooth muscle cells and the airway began to contract simultaneously. Blue arrows point to the ablated epithelial cell. *Numbers 1*, *2*, *3*, and *4* indicate epithelial cells that are ~0, 90, 90 (in opposite direction), and 140° from the ablated cell. Green dashed line represents the progress of the Ca^{2+} wave in epithelium. Yellow rectangle outlines smooth muscle cells. Red dashed line outlines the cross-sectional area of the airway before laser ablation. *C*: Ca^{2+} signaling transiently increases in each epithelial cell *(#1–4)* and Ca^{2+} wave propagates from the ablated cell to the neighboring cells indicated by the delay in Ca^{2+} signaling between the 4 selected epithelial cells (note that *#2* and *#3* rise at the same time). Ca^{2+} signaling in smooth muscle cells initiated ~10 s after laser ablation, and lasted for multiple cycles, before decaying to basal level. *D*: airway began to contract ~10 s after laser ablation. F/F_0, ratio of fluorescence intensity.

in the presence of the P2 purinoceptor receptor antagonist suramin (Supplemental Movie S13), Ip_5I, and PPADS, as well as in the presence of an ATP diphosphatase (apyrase); hence, we can rule out the ATP receptors but cannot completely rule out ATP as a soluble mediator that contributes to smooth muscle contraction induced by epithelial injury.

Acetylcholine is a potent smooth muscle constrictor (12), which could be released from intact nerves in lung tissue slices (71) or from damaged epithelial cells (51, 64, 67). To investigate the role of acetylcholine in mediating the epithelial-smooth muscle cell communication, we used atropine, an inhibitor of muscarinic acetylcholine receptors, to block the acetylcholine receptors on smooth muscle cells. We found that inhibition of acetylcholine receptors with 1 μM atropine did not block the laser ablation induced airway contraction (Fig. 5*B*). As a positive control, 1 μM atropine completely blocked the airway contraction induced by 1 μM acetylcholine (data not shown).

DISCUSSION

Epithelial injury and airway hyperresponsiveness are prominent features of asthma (5, 30, 76, 83); however, the relationship between them is largely unknown, which potentially limits the development of novel therapeutics for asthma. We hypothesized that epithelial injury could induce airway narrowing. By combining ex vivo lung tissue slices and a laser ablation technique, we precisely ruptured single epithelial cells in small airways and found that local epithelium injury induced a global airway constriction through Ca^{2+}-dependent smooth muscle shortening. Soluble mediators released from the wounded epithelial cell triggered the smooth muscle contraction. To our knowledge, this is the first demonstra-

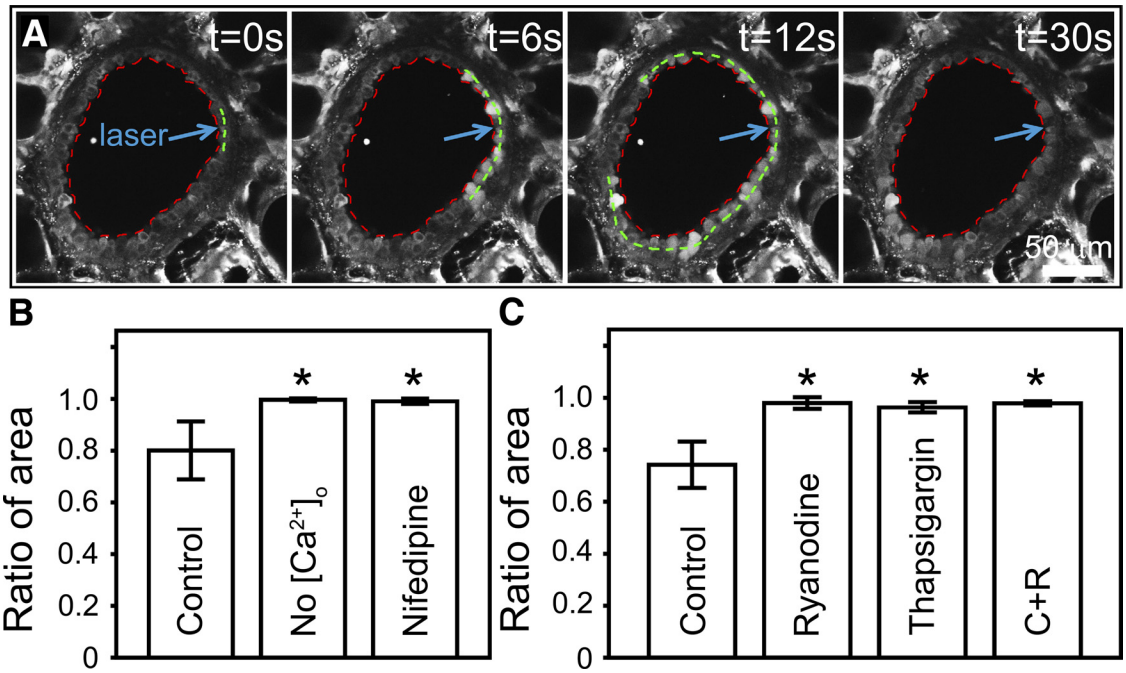

Fig. 4. Laser-induced airway contraction is Ca^{2+}-dependent. *A*: removal of extracellular Ca^{2+} blocked the Ca^{2+} oscillations in smooth muscle cells and airway contraction induced by laser ablation of single epithelial cells but did not inhibit the Ca^{2+} wave in the epithelium. Blue arrows point to the ablated epithelial cell. Green dashed line represents the progress of the Ca^{2+} wave in the epithelium. Red dashed line outlines the cross-sectional area of the airway before laser ablation. *B*: statistical tests demonstrate that removal of extracellular Ca^{2+} ($[Ca^{2+}]_o$) or inhibition of L-type voltage-dependent Ca^{2+} channels with 10 μM nifedipine significantly blocked the airway contraction induced by laser ablation of single epithelial cells. *C*: statistical tests demonstrate that inhibition of intracellular Ca^{2+} with 50 μM ryanodine, 10 μM thapsigargin, or a combination of 20 mM caffeine (C) and 50 μM ryanodine (R) significantly blocked the airway contraction induced by laser ablation of single epithelial cells. The ratio of lumen area was measured 1 min after laser ablation in treated airways (n = 5–7 airways from a minimum of 2 different rats for each condition).

tion that local epithelial injury can induce rapid and global airway constriction.

Smooth muscle contraction, a significant contributor to airway hyperresponsiveness, can be activated by two major systems: neurotransmitters from nerve terminals or inflammatory mediators from neighboring cells (4, 60). Neurotransmitters arise from the cholinergic system in which acetylcholine is released from parasympathetic nerves (20, 45) and the nonadrenergic noncholinergic system in which neurotransmitters such as substance P and ATP induce airway smooth muscle contraction (7, 11, 13, 52). Inflammatory mediators include histamine, growth factors, and cytokines released from inflammatory or resident cells (22). In contrast to neurotransmitters released from afferent nerve terminals innervating smooth muscle cells, inflammatory mediators from epithelial or other neighboring cells need to diffuse over relatively long distances (~10–100 μm) to reach smooth muscle cells (60). Thus it is largely unknown whether the concentration of soluble mediators produced by inflammatory or resident cells in asthmatics is high enough to induce smooth muscle contraction.

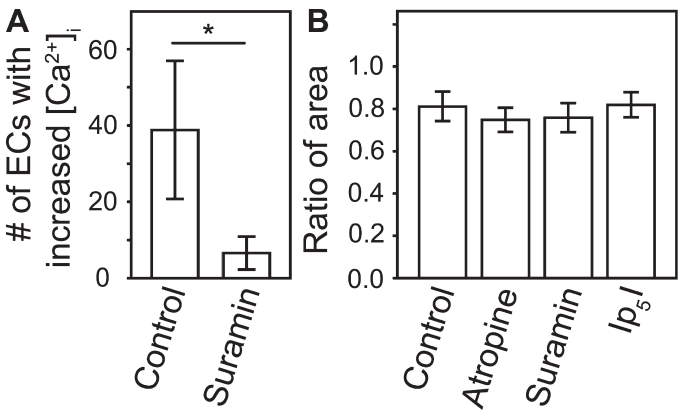

Fig. 5. *A*: 300 μM suramin, a nonselective P2 purinergic antagonist, significantly blocked the Ca^{2+} wave in epithelium. *B*: 300 μM suramin, 10 μM Ip_5I, and 1 μM atropine did not inhibit the airway contraction induced by epithelial cell injury (n = 4–5 airways from a minimum of 2 different rats for each condition).

As a physical barrier between underlying tissue and the outside environment, the airway epithelium can impact smooth muscle function. First, the integrity of epithelium prevents the direct exposure of smooth muscle cells to inhaled particles such as toxins and pollutants. Second, airway epithelial cells express receptors on their apical surface to sense environmental cues and can then transmit these signals to underlying tissue by secreting and releasing soluble mediators. For example, it has been suggested that airway epithelium modulates smooth muscle tone by epithelium-derived relaxing factors, which decrease the contractile response of tracheal smooth muscle to agonists such as acetylcholine, histamine, prostaglandin F2α, and 5-hydroxytryptamine (3, 8, 27). Third, the airway epithelium may affect fibroblasts or smooth muscle cells by direct physical contact through the epithelial-mesenchymal trophic unit (10, 35, 77). Fourth, epithelial injury induced by chemical or physical stimuli triggers wound healing responses, which increases smooth muscle proliferation (55).

To investigate the role of epithelial cell rupture in airway constriction, we used a laser ablation technique to disrupt individual airway epithelial cells in small airways and found that rupture of the apical membrane of single epithelial cells induced airway contraction to ~70% of the original cross-sectional area within 40 s. Laser ablation of epithelial cells could induce airway constriction through three possible mechanisms: *1*) laser ablation of single epithelial cells could trigger the contraction of the epithelium itself; *2*) the laser itself could activate smooth muscle contraction by transiently opening smooth muscle cell membrane receptors or stretching smooth muscle cells via photomechanical processes such as stress wave emission or cavitation bubble dynamics; and *3*) laser ablation of single epithelial cells could release soluble mediators from epithelial cells that diffuse to the smooth muscle layer and stimulate smooth muscle contraction.

The first model suggests that the epithelium is contractile. It has been shown that epithelial cells actively contract by reorganizing F-actin cytoskeleton in response to mechanical wounding in embryonic tissue (26, 43, 75). However, we did not find any contraction of epithelial cells close to the wounded cell and the gap created by the ablated cell remained after laser ablation (Fig. 3*B*), suggesting that laser ablation did not induce airway epithelial cell contraction.

The second model suggests that the laser itself activates smooth muscle cells, and only smooth muscle cells are involved in the laser-induced airway contraction. Our results provide evidence indicating that the laser itself did not induce airway smooth muscle contraction. First, we focused the laser on the apical membrane of epithelial cells, which is the farthest distance from the underlying smooth muscle cells, and we used minimal laser energy to rupture the membrane to avoid damage to neighboring cells. The latter was confirmed by propidium iodide staining. Second, we ruled out the involvement of ROS in the laser-induced airway constriction. Third, stretch of the airway wall with a blunt glass pipette, which deforms both epithelial and underlying smooth muscle cells, did not induce airway contraction, suggesting that the laser did not induce airway constriction through smooth muscle stretch.

The third model suggests that both epithelial and smooth muscle cells are involved in laser-induced airway contraction. The following results support the model that epithelial cells release soluble mediators to trigger smooth muscle contraction. First, mechanical rupture of an epithelial cell using a sharp glass micropipette triggered airway contraction, suggesting that soluble mediators released from the wounded epithelial cell stimulate airway contraction. Second, epithelial cell debris from cultured primary human small airway epithelial cells induced a similar airway contraction, confirming that soluble mediators contained in epithelial cells are capable of inducing airway contraction. Third, laser ablation of single epithelial cells immediately triggered Ca^{2+} wave in epithelial cells but did not induce airway contraction at the same time. After ~10 s, the airway started to contract, which corresponded to Ca^{2+} oscillations in the smooth muscle cells. Finally, the distance separating the epithelium and airway smooth muscle is ~25 μm (Fig. 3*B*). The characteristic time of diffusion can be estimated by the square of the diffusion distance divided by the molecular diffusion coefficient of the molecule of interest in tissue ($\sim 10^{-6}$ cm^2/s for small molecules; Ref. 29). Hence, the characteristic time of diffusion for an epithelial-derived compound to reach the smooth muscle is ~6 s, consistent with our results.

Our study shows that disruption of a single epithelial cell triggers Ca^{2+} waves in both epithelial and smooth muscle cells. The Ca^{2+} oscillations in smooth muscle cells are mediated by the calcium-induced calcium release mechanism in which Ca^{2+} influx stimulates internal Ca^{2+} release through ryanodine receptors. In contrast, the Ca^{2+} wave in epithelial cells is not dependent on Ca^{2+} influx and ryanodine receptors but is dependent on intracellular Ca^{2+} since the Ca^{2+} wave is blocked by inhibiting sarco(endo)plasmic reticulum Ca^{2+}-ATPase with thapsigargin.

To identify the nature of soluble mediators, we investigated the role of ATP and acetylcholine in mediating the epithelial-smooth muscle cell communication. We found that inhibition of P2 purinoceptor receptors significantly blocked the Ca^{2+} wave in epithelial cells but did not impact Ca^{2+} signaling in smooth muscle cells; however, neither P2 purinoceptor receptor antagonists such as suramin, Ip_5I, and PPADS, nor an ATP diphosphatase such as apyrase blocked the ATP-induced airway contraction. These results indicate that *1*) the Ca^{2+} wave in epithelium is dependent on ATP; *2*) P2 purinoceptor receptors on smooth muscle cells are not involved in smooth muscle contraction induced by epithelial injury, but we cannot completely rule out ATP as a soluble mediator; *3*) the Ca^{2+} wave in epithelium is not required to induce smooth muscle contraction; and *4*) soluble mediator(s) from the damaged epithelial cells are sufficient to activate neighboring smooth muscle cells. We also found that inhibition of muscarinic acetylcholine receptors did not block the airway contraction induced by single epithelial cell injury, which indicates that acetylcholine released either from surrounding nerves or damaged epithelial cells is not involved in the epithelial-smooth muscle cell communication.

Epithelium damage is one of the hallmarks of asthmatic airways (5, 83). However, the effect of epithelial injury on smooth muscle contractility is not well understood. Our results were surprising in that a local injury had such a global impact on the airway caliber. Pathological studies (5, 78) suggest that large numbers of epithelial cells are potentially damaged or denuded in asthmatics. It is possible that the denuded epithelium observed in histological specimens is artifact (65); nonetheless, it is clear that injury of multiple epithelial cells is not necessary to induce contraction. Instead, our data suggest that a relatively small concentration of soluble mediator from a single cell can activate a sequence of events that result in Ca^{2+} signaling in the smooth muscle and airway contraction. This result highlights the potential importance of a healthy and intact epithelium to maintain airway caliber.

Photolysis of caged compounds such as IP_3 or Ca^{2+} is commonly applied to locally induce Ca^{2+} signaling (16, 23, 32, 84). However, this technique requires caged compounds to be preloaded into cells. Our results demonstrate that laser ablation could be used to generate spatially controlled Ca^{2+} signaling in single cells embedded in three-dimensional tissue, which provides a novel method to study Ca^{2+}-dependent cell to cell communication.

As an ex vivo model, the lung tissue slice preparation has several advantages (e.g., maintaining the normal tissue structure separating the epithelium and smooth muscle) but should be used with some caution. For example, both propidium

iodide staining (Supplemental Movie S4) and the live/dead assay (Supplemental Movie S14) show that although most epithelial cells are healthy, there are a significant number of dead cells on the cut surfaces of the lung tissue slices due to trauma during tissue slicing. The dead cells may provide a pathway, which does not exist in vivo, for soluble mediators to diffuse. Furthermore, the small airways used in the lung tissue slice preparation (250-μm thickness) are essentially a short cylindrical tube that is 250 μm in length. It is possible that soluble mediators released from a single damaged epithelial cell reach the smooth muscle cells by diffusing through the lumen area (longitudinal direction) instead of diffusing through the thin extracellular matrix between epithelium and smooth muscle cells (radial direction). Thus, in our experiments, to prevent the diffusion through dead cells or longitudinally through the airway lumen, we targeted single epithelial cells that were ~75 μm from the bottom surface in the z-direction (Fig. 1*A*), where epithelial cells near the ablated cells (in *x*, *y*, and *z* planes) are healthy (Supplemental Movie S4 and S14). From this location, soluble mediators from the damaged epithelial cells need to diffuse ~25 μm to reach the smooth muscle layer by diffusing radially through extracellular matrix between epithelium and smooth muscle cells. In contrast, mediators would need to diffuse an additional 75 μm by diffusing initially longitudinally through the lumen and then radially 25 μm. For solely radial diffusion, we estimated in the text previously the characteristic time would be 6 s. For initial longitudinal diffusion (75 μm) and then radial diffusion (25 μm), the distance is four times longer, but the medium is essentially water, which has a molecular diffusivity that is approximately three times larger (29). Thus the characteristic time would be 16/3 (4 squared divided by 3, or 5.3) times the time of radial diffusion alone through the matrix. This would be >30 s, which is not consistent with the time delay (~10 s) we measured in our experiments (Supplemental Movie S8).

In conclusion, laser ablation of single epithelial cells reproducibly induced airway contraction to ~70% of the original cross-sectional area within 40 s. Single epithelial cell injury triggered a single instantaneous Ca^{2+} wave in the epithelium and released soluble mediators to underlying smooth muscle cells. The soluble mediators evoked multiple Ca^{2+}oscillations in smooth muscle cells that induced global airway contraction. Epithelial injury can induce airway contraction and may contribute to airway hyperresponsiveness in asthmatics.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: J.Z., M.B.A.-E., E.B., and S.C.G. conception and design of research; J.Z. and M.B.A.-E. performed experiments; J.Z. analyzed data; J.Z., M.B.A.-E., E.B., and S.C.G. interpreted results of experiments; J.Z. and S.C.G. prepared figures; J.Z. and S.C.G. drafted manuscript; J.Z., M.B.A.-E., E.B., and S.C.G. edited and revised manuscript; J.Z., M.B.A.-E., E.B., and S.C.G. approved final version of manuscript.

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