Adenosine A_1 and Prostaglandin EP_3 Receptors Mediate Global Airway Contraction after Local Epithelial Injury

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Epithelial injury and airway hyperresponsiveness are prominent features of asthma. We have previously demonstrated that laser ablation of single epithelial cells immediately induces global airway constriction through Ca^{2+}-dependent smooth muscle shortening. The response is mediated by soluble mediators released from wounded single epithelial cells; however, the soluble mediators and signaling mechanisms have not been identified. In this study, we investigated the nature of the epithelial-derived soluble mediators and the associated signaling pathways that lead to the L-type voltage-dependent Ca^{2+} channel (VGCC)–mediated Ca^{2+} influx. We found that inhibition of adenosine A_1 receptors (or removal of adenosine with adenosine deaminase), cyclooxygenase (COX)-2 or EP_3 receptors, epidermal growth factor receptor (EGFR), or platelet-derived growth factor receptor (PDGFR) all significantly blocked Ca^{2+} oscillations in smooth muscle cells and airway contraction induced by local epithelial injury. Using selective agonists to activate the receptors in the presence and absence of selective receptor antagonists, we found that adenosine activated the signaling pathway $A_1R \rightarrow EGFR/PDGFR \rightarrow COX\text{-}2 \rightarrow EP_3 \rightarrow VGCCs \rightarrow$calcium-induced calcium release, leading to intracellular Ca^{2+} oscillations in airway smooth muscle cells and airway constriction.

Keywords: ATP; epidermal growth factor receptor; platelet-derived growth factor receptor; cyclooxygenase-2; L-type voltage-dependent Ca^{2+} channels

CLINICAL RELEVANCE

Our studies provide direct evidence that local epithelial injury could contribute to airway hyperresponsiveness in subjects with asthma, and provides potentially new pharmacological targets for asthma treatment.

We have recently demonstrated that laser ablation of a single epithelial cell reproducibly induces rapid and global airway constriction (1). The dynamics of the response suggested that local epithelial injury released a soluble mediator(s) that was transported to underlying smooth muscle cells by diffusion. The soluble mediator(s) evoked multiple Ca^{2+} oscillations in smooth muscle cells by stimulating L-type voltage-dependent Ca^{2+} channels (VGCCs), thus increasing intracellular Ca^{2+} levels via the calcium-induced calcium release (CICR) mechanism. In this study, we investigated the specific nature of the soluble mediator(s) and signaling pathway(s) underlying the VGCC-mediated Ca^{2+} influx.

In response to mechanical stimulation, ATP is released from airway epithelial cells and stimulates Ca^{2+} waves in the epithelium (2–4); however, the effects of local epithelial injury–induced ATP on airway caliber have not been studied. The level of intracellular ATP is high (millimolar range) for metabolism, but is extremely low in the extracellular space, where it can function as a signaling molecule (5). Extracellular ATP activates two subtypes of purinergic receptors, P2X and P2Y, and both of them are expressed on airway epithelial and smooth muscle cells (6). The P2X receptors are ligand-gated ion channels that mediate Ca^{2+} and Na^{+} influx, and P2Y receptors are G protein–coupled receptors that regulate phospholipase C pathway, leading to IP_3 production and intracellular Ca^{2+} release (6). Because ATP has been shown to stimulate small airway contraction in mouse lung tissue slices by activating P2Y receptors (7), it was a likely candidate to be the soluble mediator(s) involved in local epithelial injury–induced smooth muscle contraction. However, in our previous studies, we found that inhibition of P2 purinoceptor receptors did not block Ca^{2+} signaling in smooth muscle cells and airway contraction induced by local epithelial injury. Furthermore, inhibition of P2 purinoceptor receptors or neutralizing ATP with apyrase did not block extracellular ATP–induced airway contraction. Thus, we previously ruled out the involvement of P2 purinoceptor receptors on smooth muscle cells in local epithelial injury–induced airway contraction, but we could not completely rule out ATP as a soluble mediator (1).

Exogenous ATP is rapidly converted to its metabolic products, such as adenosine monophosphate (AMP), ADP, and adenosine by ecto-apyrase enzymes expressed on the cell surface (8). Thus, ATP released from wounded single epithelial cells could potentially stimulate adenosine receptors after degradation to AMP (9) or adenosine (6, 10). Adenosine receptors are G protein–coupled receptors and have four subtypes: A_1, A_{2A}, A_{2B}, and A_3 receptors. Although the four adenosine receptors are all expressed on airway smooth muscle cells (6), adenosine is traditionally considered to indirectly induce bronchoconstriction either through activating A_{2B} or A_3 receptors on mast cells or through neural nerves via A_1 receptors (11–14). However, recent studies demonstrated that adenosine could directly activate A_1 and A_{2B} receptors to regulate adenylyl cyclase on human tracheal smooth muscle cells (15), and stimulate A_1 receptors on human bronchial smooth muscle cells to increase Ca^{2+} signaling (16). Furthermore, adenosine A_1 receptors are colocalized with epidermal growth factor receptors (EGFRs) and induce the transactivation of EGFR and their downstream pathways, such as phosphoinositide 3-kinase and Src kinase in neural cells (17).

Prostanoids, which are derived from arachidonic acid, include prostaglandins (PGE_2, PGD_2, and PGF_2), prostacyclins (PGI_2), and thromboxane (18). Cyclooxygenase (COX-1 and COX-2) is an enzyme that converts arachidonic acid into the prostanoids. High levels of prostanoids in bronchoalveolar lavage fluid and

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the increased expression of COX-2 in lung tissue have been detected in subjects with asthma (18). PGE_2 is produced by airway epithelial cells (19–21) and by smooth muscle cells (22–25), and has been demonstrated to regulate airway caliber (26–28). There are four subtypes of PGE_2 receptors: EP_{1-4}. Activation of EP_2 or EP_4 receptors increases intracellular cyclic AMP level and causes relaxation of smooth muscles (29, 30), whereas activation of EP_1 receptors increases intracellular Ca^{2+} and thus causes smooth muscle contraction (23, 31, 32). EP_3 has multiple isoforms, and its activation can stimulate smooth muscle contraction through either decreasing cyclic AMP levels or increasing intracellular Ca^{2+} (30, 33).

The goal of this study was to identify the soluble mediator(s) and signaling pathway(s) mediating the local epithelial injury–induced smooth muscle contraction in rat lung tissue slices. We hypothesized that adenosine and PGE_2 were both involved in the underlying mechanisms of local epithelial injury–induced airway contraction. Our results show that ATP is released from wounded single epithelial cells, and activates the following sequence of events: $A_1R \rightarrow$EGFR/PDGFR$\rightarrow$COX-2$\rightarrow EP_3 \rightarrow$VGCCs$\rightarrow$CICR, leading to the Ca^{2+} oscillations in smooth muscle cells and airway contraction.

MATERIALS AND METHODS

Materials

Fluo-4/AM, Pluronic F-127, Hanks' balanced salt solution (HBSS), Dulbecco's modified Eagle medium, and Antibiotic-Antimycotic were purchased from Invitrogen (Carlsbad, CA). AH6809, AG18, AG1478, AG1296, and 11-deoxy-16,16-dimethyl PGE_2 (11-PGE_2) were purchased from Cayman Chemical (Ann Arbor, MI). Sulfobromophthalein, ATP, adenosine 5′-[γ-thio]triphosphate tetralithium salt (ATP-γ-S), indomethacin, L-798106, and N6-cyclopentyladenosine (CPA) were purchased from Sigma-Aldrich (St. Louis, MO). Adenosine, 9-chloro-2-(2-furanyl)-[1,2,4] triazolo[1,5-*c*]quinazolin-5-amine (CGS15943), 1-butyl-8-(hexahydro-2,5-methanopentalen-3a(1*H*)-yl)-3,7-dihydro-3-(3-hydroxypropyl)-1*H*-purine-2,6-dione (PSB36), 8-[4-[4-(4-chlorophenzyl)piperazide-1-sulfonyl) phenyl]]-1-propylxanthine (PSB603), SLV320, ZM241385, and MRS1334 were purchased from Tocris Bioscience (Ellisville, MO). Supplemented HBSS (sHBSS) was made from HBSS with Ca^{2+} and Mg^{2+} supplemented with 20 mM Hepes (pH 7.4) (34).

Because the selectivity of an inhibitor is dependent on species (e.g., human versus rat) and cell type, for each chemical compound that we used in this study, we have provided detailed information for the concentration used based on the previous studies in rat (*see* Table E1 in the online supplement). Because molecular methods, such as siRNA or lentivirus-based shRNA, to confirm the molecular mechanism in our current studies, introduce significant technical challenges in the lung tissue slice model, we applied multiple selective inhibitors to confirm our results.

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 guidelines published by the National Institutes of Health. The preparation of rat lung tissue slices has been previously described in detail (1), and the procedure is also available in the MATERIALS AND METHODS section of the online supplement.

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 sHBSS with 20 μM Fluo-4/AM, 100 μM sulfobromophthalein, and 0.2% Pluronic F-127 for 1 hour at room temperature (35). 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). Fluo-4 was excited with a 488-nm laser, and the fluorescence images (512 × 512 pixels) were collected.

Laser ablation

The procedure for femtosecond (fs) laser ablation has been previously described in detail (1). Briefly, the laser ablation was performed on the LSM 510 with an Achroplan 40×/0.8 NA water-immersion objective. A single epithelial cell was ruptured by focusing the Mode-locked Ti: Sapphire femtosecond laser beam over a triangular region of interest (~6 μm^2) that included the apical membrane of the epithelial cell. The region of interest was scanned horizontally by the femtosecond laser at 100 μs/μm. By using the "bleach control" program in the LSM 510, we were able to immediately (less than 1 second) 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 at the sample plane was ~600 mW, the pulse energy was ~7.5 nJ per pulse, and the peak power was ~37.5 kW.

Statistical 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 performed with one-way ANOVA using commercial software (SPSS v. 16; SPSS, Chicago, IL), and a P value less than 0.05 was considered statistically significant.

RESULTS

Adenosine and A_1 Receptor Mediate the Local Epithelial Injury–Induced Airway Contraction

In our previous studies, we ruled out the involvement of P2 purinoceptor receptors on smooth muscle cells in local epithelial injury–induced airway contraction; however, we did not completely rule out ATP as a soluble mediator, because inhibition of P2 purinoceptor receptors did not block the ATP-induced airway contraction (1). One explanation for these results would be activation of adenosine receptors to stimulate airway contraction from ATP metabolites, such as AMP and adenosine. To test this possibility, we inhibited the adenosine receptors with 2 μM CGS15943, a nonselective adenosine receptor antagonist (36, 37). Laser ablation of single epithelial cells induced an increase in Ca^{2+} oscillations (*see* Figure E1A in the online supplement), and airway contraction to 70% of the original cross-sectional area (Figures 1A and 1C and Movie E1). However, inhibition of adenosine receptors with CGS15943 completely blocked Ca^{2+} oscillations in smooth muscle cells and airway contraction induced by local epithelial injury, but did not block the Ca^{2+} wave in epithelial cells (Figure 1C, Figure E1B, and Movie E2). To confirm that adenosine is the soluble mediator, we incubated lung tissue slices with 5 units/ml adenosine deaminase (ADA), an enzyme that catalyzes the deamination of adenosine (38), and found that ADA significantly blocked the local epithelial injury–induced airway contraction (Figure 1C).

Because all the four subtypes of the adenosine receptors A_1, A_{2A}, A_{2B}, and A_3 receptor have been demonstrated to mediate smooth muscle contraction (5, 11–16, 39–41), we next investigated the roles of these four adenosine receptors. We found that selective inhibition of the A_1 receptor with 5–50 μM PSB36 (42, 43) or 4 μM SLV320 (44) significantly blocked Ca^{2+} oscillations in smooth muscle cells and airway contraction induced by local epithelial injury, but, again, did not block the Ca^{2+} wave in epithelial cells (Figures 1B and 1C, Figure E1C, and Movie E3). In contrast, selective inhibition of the A_{2A} receptor with 5 μM ZM241385 (45, 46), A_{2B} receptor with 10 μM PSB603 (47, 48), or A_3 receptor with 5 μM MRS1334 (49) did not block the

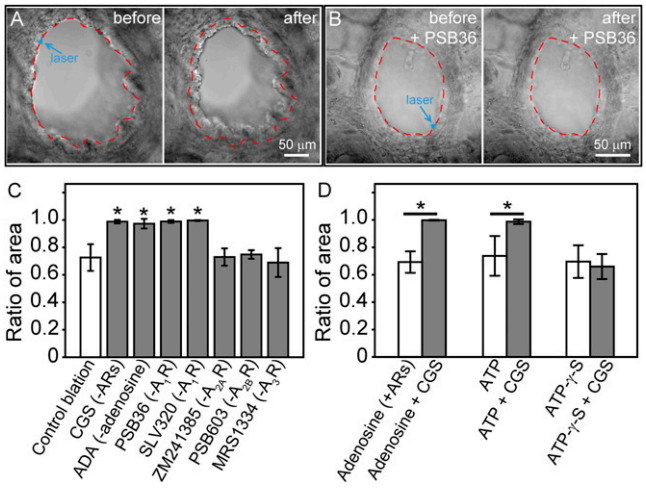

Figure 1. Adenosine released from a wounded single epithelial cell activates A_1 receptors to induce airway contraction. (*A*) Bright-field images of a small airway embedded in a lung tissue slice before and ~40 seconds after laser ablation demonstrate that damage of a single epithelial cell induces global airway contraction. *Blue arrow* points to the ablated epithelial cell, the apical membrane of which was ruptured by a pulsed femtosecond laser. *Red dashed line* outlines the lumen cross-sectional area before laser ablation. (*B*) Inhibition of A_1 receptors with 50 μM PSB36 blocked local epithelial injury–induced airway contraction. (*C*) Local epithelial injury induced airway contraction, which leads to a roughly 30% reduction of the original cross-sectional area in control airways ($n = 33$ airways from 12 rats). Statistical tests demonstrate that inhibition of adenosine receptors with 2 μM 9-chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-*c*]quinazolin-5-amine (CGS15943) (CGS, a nonselective adenosine receptor antagonist; $n = 7$ airways from 5 rats), inhibition of adenosine A_1 receptors with 5–50 μM PSB36 (50 μM for 15 min incubation time, or 5 μM for 60 min; $n = 5$ airways from 3 rats for 50 μM PSB36 treatment) or 4 μM SLV320 ($n = 9$ airways from 4 rats), or deletion of adenosine with 5 units/ml adenosine deaminase (ADA; $n = 4$ airways from 3 rats) significantly blocked the airway contraction induced by local epithelial injury, whereas inhibition of adenosine A_{2A} receptors with 5 μM ZM241385 ($n = 4$ airways from 2 rats), A_{2B} receptors with 10 μM PSB603 ($n = 6$ airways from 5 rats), or A_3 receptors with 5 μM MRS1334 ($n = 8$ airways from 6 rats) did not. (*D*) Statistical tests demonstrate that CGS15943 significantly blocked airway contraction induced by 10 μM adenosine (ADO, a nonselective adenosine receptor agonist; $n = 4$ airways from 4 rats for N6-cyclopentyladenosine [CPA] and $n = 4$ airways from 4 rats for CPA + CGS treatment) or 10 μM ATP ($n = 5$ airways from 5 rats for ATP and $n = 4$ airways from 4 rats for ATP + CGS), but not 10 μM ATP-γ-S ($n = 6$ airways from 4 rats for ATP-γ-S and $n = 5$ airways from 4 rats for ATP-γ-S + CGS). As a positive control, 25 mM KCl or 1 μM acetylcholine was used to verify the viability and contractility of airway smooth muscle cells after laser ablation experiments for each treatment. 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 between control airways and each treatment condition were performed with one-way ANOVA, and there are similar numbers of control airways for each treatment condition. $P < 0.05$ was considered statistically significant.

local epithelial injury–induced Ca^{2+} oscillations in smooth muscle cells and airway contraction (Figure 1C).

To test the role of adenosine receptors in mediating ATP-induced airway contraction, we investigated the effects of ATP, its nonhydrolyzable analog, ATP-γ-S, and adenosine on airway caliber in the presence and absence of adenosine receptor antagonist CGS15943. We found that 10 μM adenosine, ATP, or ATP-γ-S induced airway contraction (Figure 1D). Inhibition of adenosine receptors with CGS15943 significantly blocked airway contraction induced by adenosine and ATP, but not by ATP-γ-S (Figure 1D).

EP_3 Receptors Participate in the Local Epithelial Injury–Induced Airway Constriction

PGE_2 has been shown to be released by mechanical scratch of the guinea pig tracheal mucosa (19), indicating that epithelial cell damage could possibly increase PGE_2 levels in lung tissue. Thus, we investigated the role of PGE_2 in local epithelial injury–induced airway contraction by inhibiting COX-2, an enzyme that mediates PGE_2 production. We found that inhibition of COX-2 with 20 μM indomethacin, a nonselective COX inhibitor (50–52), or with 10 μM NS-398, a selective COX-2 inhibitor (24, 53, 54), completely blocked Ca^{2+} oscillations in smooth muscle cells and airway contraction induced by local epithelial injury, but did not block the Ca^{2+} wave in epithelial cells (Figure 2A, Figure E1D, and Movie E4).

Because there are four EP receptors presented on airway smooth muscle cells (55), we next attempted to determine whether a selective EP receptor(s) mediates the local epithelial injury–induced airway contraction. We found that 10–25 μM L-798106, a selective EP_3 antagonist (56–59), and 100 μM AH6809, a nonselective rat EP_1, EP_2, and EP_3 receptor inhibitor (59–61), completely blocked Ca^{2+} oscillations in smooth muscle cells and airway contraction induced by local epithelial injury, but did not block the Ca^{2+} wave in epithelial cells (Figure 2B, Figure E1E, and Movie E5).

To investigate the order in which A_1 and EP_3 receptors were activated, we assessed the effects of CPA, a selective A_1R agonist (45, 62), and 11-PGE_2, a stable synthetic analog of PGE_2 that selectively activates EP_3 receptor (61, 63), on airway caliber in the presence and absence of EP_3 receptor antagonist L-798106. We found that both 10 μM CPA and 11-PGE2 induced airway contraction (Figure 2C). Inhibition of EP_3 receptors with L-798106 significantly blocked airway contraction induced by CPA or 11-PGE_2, but not by 25 mM KCl, a VGCC agonist (Figure 2C). However, inhibition of A_1R with 50 μM PSB36 did not block airway contraction with 11-PGE_2 (Figure 2C). Thus, the role of A_1R is upstream of EP_3 and VGCC in the activation of smooth muscle contraction after airway epithelial injury.

Adenosine A_1R Activates EP_3 Receptors via EGFR/PDGFR Pathway

It has been demonstrated that adenosine A_1 receptor mediates the transactivation of the EGFR in rat cortical neurons (17), whereas activation of receptor tyrosine kinases, such as EGFR and PDGFR, could increase the production of PGE_2 in human or guinea pig tracheal smooth muscle cells (23, 24). Thus, we hypothesized that receptor tyrosine kinases are necessary for adenosine A_1R–mediated EP_3 receptor activation. To assess the role of receptor tyrosine kinases in local epithelial injury–induced airway contraction, we blocked receptor tyrosine kinases with 100 μM AG18, an inhibitor of EGFR and PDGFR (64–66), and 10 μM AG1478 (24, 67, 68) or 10 μM AG1296 (68–70), which are selective inhibitors of EGFR and PDGFR, respectively. We observed that inhibition of EGFR or PDGFR significantly blocked Ca^{2+} oscillations in smooth muscle cells and airway contraction induced by local epithelial injury, but did not block the Ca^{2+} wave in epithelial cells (Figure 3A, Figure E1F, and Movie E6).

To demonstrate whether the receptor tyrosine kinase pathway is involved in adenosine A_1R–mediated activation of EP_3 receptors, we stimulated A_1 and EP_3 receptors with CPA and 11-PGE2, respectively, in the presence and absence of EGFR inhibitor, AG1478. We found that inhibition of EGFR with 10 μM

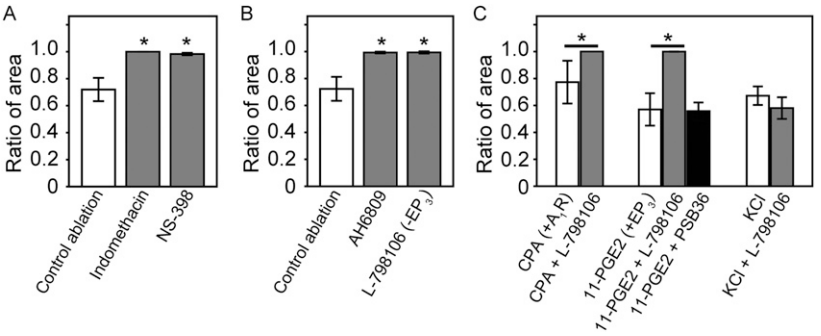

Figure 2. EP_3 receptors mediate adenosine-induced airway contraction. (*A*) Statistical tests demonstrate that inhibition of cyclooxygenase (COX)-2 with 20 μM indomethacin ($n = 5$ airways from 5 rats) or 10 μM NS-398 ($n = 4$ airways from 2 rats) completely blocked local epithelial injury–induced airway contraction ($n = 10$ control airways from nine rats). (*B*) Statistical tests demonstrate that inhibition of EP_3 receptor with 100 μM AH6809 ($n = 8$ airways from 8 rats) or 10–25 μM L-798106 (25 μM for 20 min incubation time or 10 μM for 45 min; $n = 5$ airways from 2 rats for 25 μM L-798106 treatment) completely blocked local epithelial injury–induced airway contraction ($n = 14$ control airways from ten rats). (*C*) Statistical tests demonstrate that L-798106 significantly blocked airway contraction induced by 10 μM CPA, an adenosine A_1 receptor agonist ($n = 5$ airways from 3 rats for CPA and $n = 5$ airways from 4 rats for CPA + L-798106), or 10 μM 11-prostaglandins E_2 (PGE_2), an EP_3 receptor agonist ($n = 9$ airways from 5 rats for 11-PGE_2 and $n = 5$ airways from 4 rats for 11-PGE_2 + L-798106), but not 25 mM KCl, a L-type voltage-dependent Ca^{2+} channel (VGCC) agonist ($n = 6$ airways from 4 rats for KCl and $n = 5$ airways from 4 rats for KCl + L-798106). Inhibition of A_1R with 50 μM PSB36 did not block airway contraction with 10 μM 11-PGE2 ($n = 4$ airways from 2 rats).

AG1478 significantly blocked airway contraction induced by 10 μM CPA, but not by 10 μM 11-PGE2 or 25 mM KCl (Figure 3C). Thus, the role of EP_3 and VGCC in the activation of smooth muscle contraction is downstream of EGFR.

DISCUSSION

Local epithelial injury induces airway hyperresponsiveness (1); however, the underlying mechanism has not been identified. In this study, we investigated the underlying mechanisms of the rapid (<10 s) communication between local epithelial injury and airway constriction by combining a lung tissue slice model with a femtosecond laser ablation technique. We first identified adenosine as the soluble mediator initiating local epithelial injury–induced airway contraction via A_1R activation. We then revealed a novel signaling pathway that includes the sequence $A_1R \rightarrow$EGFR/PDGFR$\rightarrow$COX-2$\rightarrow EP_3 \rightarrow$VGCCs$\rightarrow$CICR, leading to increase in Ca^{2+} oscillations in airway smooth muscle cells and initiation of airway constriction. For the first time, we show a sequential link between adenosine A_1R, receptor tyrosine kinases, including EGFR and PDGFR, and prostaglandin receptor EP_3 in airway smooth muscle cells.

ATP regulates multiple biological responses, such as airway hyperresponsiveness in the lungs (6). As an energy source, ATP is maintained at a very high level in the cytoplasm of airway epithelial cells. Upon epithelial injury, the local concentration of ATP can rapidly increase to 125 μM (38) and initiate a Ca^{2+} wave in airway epithelium by activating P2Y receptors on epithelial cells (4). However, the effects of local epithelial injury–induced ATP release on airway caliber have not been studied. In this study, we found that local epithelial injury–induced ATP activated both airway epithelial and smooth muscle cells. Locally, ATP activates P2 purinergic receptors on neighboring epithelial cells; however, over the time (~5–10 s [1]) it takes to diffuse to the underlying smooth muscle, ATP is rapidly (<1 s [71]) degraded to adenosine, leading to the stimulation of adenosine A_1 receptors on smooth muscle cells (Figure 4). The hypothesis that ATP is rapidly degraded into adenosine is supported by the results that ATP-induced airway contraction is blocked by inhibition of adenosine receptors (Figure 1D), but not by inhibition of P2 purinergic receptors (1). In the present study, we confirmed the role of adenosine in the local epithelial injury–induced airway contraction by both inhibiting A_1 receptors and neutralization of adenosine with ADA; however, we cannot rule out the involvement of AMP, because AMP, which has been shown to activate A_1 receptors (9), might also be decreased by ADA.

ATP has been demonstrated to induce airway smooth muscle contraction through either directly activating P2X or P2Y on mouse airway smooth muscle cells (7) or indirectly stimulating P2Y receptors on epithelial cells, which release prostaglandins in guinea pig trachea (20). We can eliminate the direct effect of ATP on airway smooth muscle cells, because inhibition of P2X or P2Y on smooth muscle cells did not block the local epithelial injury–induced smooth muscle contraction (1). Furthermore, we can rule out the indirect effect of ATP on airway epithelial cells, because inhibition of P2 purinergic receptors on epithelial cells significantly decreased the Ca^{2+} wave in the epithelium, but did not block the local epithelial injury–induced airway contraction (1). Thus, we have identified a novel pathway in which adenosine derived from local epithelial injury–released ATP stimulates A_1 receptors on smooth muscle cells to initiate airway contraction in rat lung tissue slices.

The levels of adenosine in bronchoalveolar lavage fluid are increased in asthma, and hyperresponsiveness to adenosine is a hallmark of asthma (72). Adenosine is traditionally thought to induce airway contraction indirectly by activation of adenosine receptors, either on neural nerves or on mast cells, which release acetylcholine and prostaglandins, respectively, to induce smooth muscle cell contraction (11). However, we ruled out the involvement of sensory nerves in adenosine-induced airway contraction (11–14), because inhibition of acetylcholine M2 receptors with atropine did

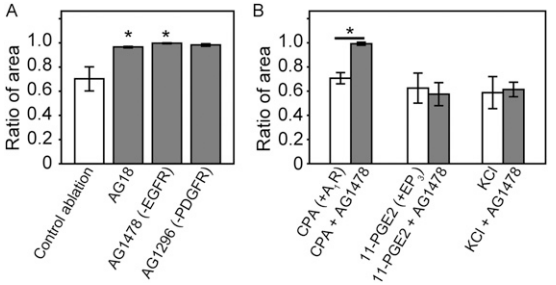

Figure 3. Epidermal growth factor receptor (EGFR)/platelet-derived growth factor receptor (PDGFR) mediates A_1R-induced activation of EP_3 receptors. (*A*) Statistical tests demonstrate that inhibition of EGFR and PDGFR with 100 μM AG18 ($n = 4$ airways from 3 rats), or inhibition of EGFR or PDGFR with 10 μM AG1478 ($n = 5$ airways from 3 rats) and 10 μM AG1296 ($n = 4$ airways from 2 rats), respectively, significantly blocked local epithelial injury–induced airway contraction ($n = 9$ control airways from 5 rats). (*B*) AG1478 significantly blocked airway contraction induced by 10 μM CPA ($n = 5$ airways from 2 rats for CPA and $n = 6$ airways from 3 rats for CPA + AG1478), but not 10 μM 11-PGE_2 ($n = 6$ airways from 4 rats for 11-PGE_2 and $n = 6$ airways from 4 rats for 11-PGE_2 + AG1478) or 25 mM KCl ($n = 6$ airways from 5 rats for KCl and $n = 6$ airways from 3 rats for KCl + AG1478).

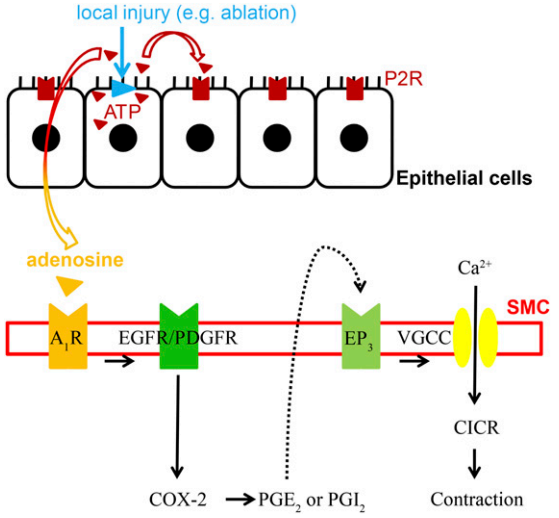

Figure 4. Schematic diagram illustrating the sequential pathway involved in local epithelial injury–induced airway contraction. A single wounded epithelial cell releases ATP that activates P2 purinergic receptors on epithelial cells, and rapidly degrades into adenosine. Adenosine diffuses to the underlying smooth muscle and stimulates adenosine A_1 receptors. The stimulated A_1 receptors activate EGFRs/PDGFRs, which increase the activity of COX-2. COX-2 increases PGE_2 or prostacyclin (PGI_2) production, which, in turn, activates EP_3 receptors. Activated EP_3 receptors stimulate VGCCs, which increase intracellular Ca^{2+} by the Ca^{2+}-induced Ca^{2+} release (CICR) mechanism. SMC, smooth muscle cell.

not block the local epithelial injury–induced airway contraction (1). We also ruled out the involvement of mast cell degranulation via A_{2B} receptor, because neither inhibition of A_{2B} receptor (Figure 1C) nor inhibition of mast cell degranulation with 300 μM cromolyn sodium (data not shown) blocked the local epithelial injury–induced airway contraction. Thus, we conclude that adenosine directly induces airway smooth muscle contraction by activation of A_1 receptors on the airway smooth muscle cells. Although adenosine has been shown to activate A_1 and A_{2B} receptors on human airway smooth muscle cells and regulate adenylyl cyclase and Ca^{2+} signaling (15, 16), the underlying mechanism leading to mobilization of Ca^{2+} is not clear.

In this study, we show that inhibition of EGFR/PDGFR completely blocks the local epithelial injury–induced airway contraction. We further demonstrate that inhibition of EGFR significantly blocks the A_1R agonist–induced airway contraction, but not EP_3 agonist–induced contraction (Figure 3C). The results indicate that receptor tyrosine kinases, including EGFR and PDGFR, are necessary for A_1R-mediated EP_3 activation. Our results are consistent with those of previous studies in which adenosine A_1 receptors mediated the transactivation of EGFRs in neural cells (17). Because receptor tyrosine kinases regulate many cellular functions, such as cell migration, differentiation, proliferation, apoptosis, and inflammation (73, 74), it is possible that local epithelial injury–induced receptor tyrosine kinase activation may have an even broader impact on pulmonary pathology. Multiple signaling pathways could be involved in EGFR/PDGFR–induced COX-2 production, such as phosphoinositide 3-kinase/Akt/NF-κB (24), mitogen-activated protein kinase kinase/mitogen-activated protein kinase (23, 75), or c-Src (76), and further study is needed to reveal the downstream signaling pathways.

Inhibition of COX-2 with indomethacin and NS-398 completely blocked the local epithelial injury–induced airway contraction, demonstrating the involvement of prostanoids in this process. To confirm this, we found that inhibition of EP_3 receptors also blocked the airway contraction induced by local epithelial injury. Although PGE_2 is traditionally considered an agonist of EP_3 receptors, PGI_2 has recently been shown to activate EP_3 receptors (59), indicating that either PGE_2 or PGI_2 could be released upon local epithelial injury. Our results are consistent with the studies in which activation of receptor tyrosine kinases leads to COX-2 expression (75–77) and PGE_2 generation (24). Our results are also consistent with the studies showing that smooth muscle cell contraction can be stimulated by self-generated prostanoids (23).

VGCC-mediated Ca^{2+} influx induces a large amount of intracellular Ca^{2+} release, which regulates numerous cellular functions, including smooth muscle contraction. In our previous study, we demonstrated that inhibition of VGCCs with nifedipine completely blocked local epithelial injury–induced airway contraction. We further showed that inhibition of A_1 and EP_3 receptors did not block KCl-induced airway contraction (Figures 2C and 3C), whereas inhibition of VGCCs blocked A_1 and EP_3 receptor agonist-induced airway contraction (data not shown). These results suggest that A_1R-, EGFR-, and EP_3 receptor–mediated pathways are upstream and dependent on VGCCs.

We acknowledge the use of relatively high concentrations of some chemical inhibitors in comparison to reported negative log dissociation constants or IC_{50} (Table E1), and thus off-target effects for one or more of the compounds are possible. However, in our present study, we report a series of compelling observations (multiple agonists and antagonists for each receptor) that are all consistent with the revealed molecular pathway underlying local epithelial injury–induced airway contraction. Thus, it is essentially impossible that all potential off-target effects would lead to the same set of conclusions.

In conclusion, we have identified a novel sequence of events that provides the underlying mechanism by which local airway epithelial injury can induce global airway smooth muscle contraction. Airway epithelial cell injury releases ATP, which is rapidly degraded to adenosine. Adenosine can diffuse to the underlying smooth muscle and initiates the activation of A_1R, EGFR and PDGFR, COX-2, EP_3, and VGCCs. Our studies provide direct evidence that local epithelial injury could contribute to airway hyperresponsiveness in subjects with asthma, and provides potentially new pharmacological targets for asthma treatment.

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