Adenosine Regulation of Cystic Fibrosis Transmembrane Conductance Regulator through Prostenoids in Airway Epithelia

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Cystic fibrosis is caused by dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) protein, leading to altered ion transport, chronic infection, and excessive inflammation. Here we investigated regulation of CFTR in airway cell monolayers by adenosine, adenosine receptors, and arachidonic acid. Our studies demonstrate that the A2B adenosine receptor is expressed at high levels relative to the other adenosine receptor subtypes, with a characteristic low-affinity profile for adenosine-stimulated CFTR Cl^- currents in both Calu-3 cells and CFBE41o- airway cell monolayers stably transduced with wild-type CFTR. The levels of adenosine found in sputum from patients with cystic fibrosis with moderate to severe lung disease stimulated apical prostaglandin release in Calu-3 and CFBE41o- cells, implicating adenosine regulation of phospholipase A2 (PLA2) activity. A2B adenosine receptor and arachidonic acid stimulation produced CFTR-dependent currents in airway monolayers and increased cAMP levels that were sensitive to cyclooxygenase inhibition. Arachidonic acid demonstrated dual regulation of CFTR, stimulating CFTR and Cl^- currents in intact airway monolayers, and potently inhibiting PKA-activated Cl^- currents in excised membrane patches. Cl^- currents produced by arachidonic acid were sensitive to inhibition of PKA, cyclooxygenase, and 5-lipoxygenase. Together, the results provide a converging mechanism to link regulation of CFTR and airway cell inflammation through adenosine and adenosine receptors.

Keywords: adenosine receptors; arachidonic acid; cystic fibrosis; inflammation; Phospholipase A2

Cystic fibrosis (CF) results from mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator, or CFTR. The protein product is a traffic ATPase and Cl^- channel that localizes to the apical membrane of airway epithelia. CFTR dysfunction leads to many abnormalities in the airways, including loss of Cl^- permeability, increased Na^+ absorption, dessication of the airway surface liquid (ASL), and an enhanced airway epithelial inflammatory response to bacterial and viral infection (1–3). Work from a variety of laboratories has demonstrated abnormalities in inflammatory signaling in the CF airway, including increased production of IL-8, decreased levels of IL-10, chronic luminal inflammation with an acute granulocyte influx (dominated by neutrophils), and more recently, defects in lipoxin limitation of acute inflammation (4–8). To date, few studies of CF inflammation have identified direct links between dysregulated airway ion transport and inflammation in the disease.

A recent series of studies completed by Freedman and colleagues has helped to define abnormalities in membrane lipids in CF-affected tissues of mice and human subjects, including increased ratios of arachidonic acid (AA): docohexanoic acid (DHA) in CF serum, airways, and rectal biopsies (9–11). AA is stored in cell membranes, and is released from the sn-2 position of membrane lipids by various phospholipase A2 (PLA2) isoforms. It is the parent molecule of prostanoids and thromboxanes following cyclooxygenase and prostaglandin synthase metabolism, and leukotrienes that are the product of 5-lipoxygenase activity and distal enzymes in the leukotriene synthesis pathway (12, 13). These eicosanoids signal through unique G protein–coupled receptors (GPCRs), which elicit diverse responses that are cell type and tissue specific. Many of these molecules regulate inflammation, and serve important functions in granulocyte chemotaxis, aggregation and degranulation, increased tissue and vascular permeability, induction of bronchoconstriction, and mucus production.

Adenosine (Ado) is an important regulator of CFTR in the airways through stimulation of Ado receptors, and serves a vital function regulating the ASL depth and composition (3, 14, 15). Ado is a ubiquitous signaling molecule, controlling diverse functions in many tissues, including inflammation in tissue compartments and epithelial ion transport (16). Recent studies by Blackburn and colleagues have highlighted potent proinflammatory effects produced by high levels of Ado and stimulation of Ado receptors in the mouse lung (17, 18). Work from our laboratory has suggested that A2B Ado receptor (A2B AR) stimulation activates PLA2, which contributes to CFTR regulation *in vitro* and *in vivo* (19–21).

In this study, we investigated regulation of CFTR and AA metabolism by Ado and A2B ARs. Our studies point toward a direct link between regulation of CFTR activity and AA production by Ado and A2B ARs, and provide a plausible mechanism connecting regulation of CFTR function to airway inflammation through Ado-dependent signaling pathways in airway cells.

MATERIALS AND METHODS

Growth and Propagation of Cells (Airway and Nonairway Cell Types)

Wild-type and ΔF508 CFTR cDNA was stably introduced into HeLa and CFBE41o- cells using TranzVector (Tranzyme, Inc., Birmingham, AL). TranzVector system represents an HIV-based lentiviral vector with unique safety features as described (22). To generate vector stock, CFTR cDNA was first cloned into the gene transfer component under the control of the human cytomegalovirus (hCMV) promoter. Expression of CFTR was also coupled to the puromycin-N-acetyltransferase gene via the internal ribosomal entry site of encephalomyocarditis virus, allowing for rapid selection of cells expressing CFTR in media containing puromycin. HeLa and CFBE41o- cells were transduced at multiplicity of infection of one followed by puromycin (4 μg/ml) selection. Puromycin-resistant cells were expanded to form a pool of stable CFTR

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expressors. Cells were seeded onto fibronectin-coated Costar 6.5-mm inserts (Costar, Cambridge, MA) at a concentration of $\sim 5 \times 10^6$ cells/insert, and grown for 5–7 d until confluent. Cells were then switched to an air–liquid interface for 48 h before study. For some experiments, CFBE41o- ΔF508 cells were grown at 27°C for 48 h before study in Ussing chambers. This maneuver has been shown to rescue substantial amounts of ΔF508 CFTR to the cell membrane ($\sim$ surface CFTR levels in wtCFTR transduced CFBE41o- cells), with retention of cAMP signaling pathways (23). Calu-3 cells (wtCFTR-expressing, serous cell type) were purchased from ATCC, and grown in MEM + 10% FBS and nonessential amino acids (Gibco, Grand Island, NY). 16HBE cells were the generous gift of Dr. Dieter Gruenert (California Pacific Medical Center, San Francisco, CA), and were propagated in MEM + 10% FBS. Primary human airway cells were obtained from surgical (post–lung transplant) specimens, derived from normal donor or CF recipient bronchial remnant sections. Cells were isolated as previously described (20). Tissue was disassociated by pronase enzyme digestion (1.5 mg/ml pronase, 0.1 mg/ml DNase in calcium- and magnesium-free MEM at 4°C for 48–96 h). Digestion was then stopped with FBS (10% vol/vol), and cells were pelleted by centrifugation ($200 \times g$, 5 min) and resuspended in DMEM/F12 media + 2% Ultroser G + insulin (10 μg/ml), transferrin (10 μg/ml), selenium (10 ng/ml), hydrocortosone (0.5 μg/ml), T_3-triiodotyromine (20 ng/ml), epithermal growth factor (25 ng/ml), and endothelial growth supplement (30 μg/ml). Cells were seeded onto Transwell filters (0.33 cm^2) at a density of $\sim 1 \times 10^6$ per cm^2. After 4–6 d of incubation, an air interface at the apical membrane was established, and the media bathing the basolateral surface was changed every 48 h. Measurement of transepithelial resistance was followed until maturation ($\sim$ 2 wk).

Ussing Chamber Studies in Airway Cell Monolayers

Inserts were mounted in Ussing chambers, and I_{sc} was measured under voltage clamp conditions as previously described (20, 23). Briefly, cells grown at an air–liquid interface were mounted in modified Ussing chambers (Jim's Instruments, Iowa City, IA), and initially bathed on both sides with identical Ringers solutions containing (in mM) 115 NaCl, 25 $NaHCO_3$, 2.4 KH_2PO_4, 1.24 K_2HPO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, and 10 D-glucose (pH 7.4). Bath solutions were vigorously stirred and gassed with 95%O_2:5% CO_2. Solutions and chambers were maintained at 37°C. Short circuit current (I_{sc}) measurements were obtained by using an epithelial voltage clamp (University of Iowa Bioengineering, Iowa City, IA). A 3-mV pulse of 1 s duration was imposed every 100 s to monitor resistance, which was calculated using Ohm's law. To measure stimulated I_{sc}, the mucosal bathing solution was changed to a low Cl^- solution containing (in mM) 1.2 NaCl, 115 Na gluconate, and all other components as above plus 100 μM amiloride. Increasing agonist concentrations were added to the bathing solutions as indicated (minimum 5 min of observation at each concentration). Unless otherwise noted, time points were taken at 500-s intervals. 200 μM glybenclamide was added to the mucosal bathing solution at the end of experiments to block CFTR-dependent I_{sc}.

SPQ Studies of Halide Efflux in Hela Cells

HeLa cells stably expressing wt CFTR were studied with the halide quenched dye 6-methoxy-N-(3-sulfopropyl)-quinolinium (SPQ; Molecular Probes Inc., Eugene, OR) as previously described (23, 24). Briefly, cells were seeded at $\sim 5 \times 10^5$ cells/coverslip and grown in DMEM + 10% FBS at 37°C for 24–48 h. On the day of study, cells were loaded with hypotonic SPQ (3 mM) for 10 min (37°C), and then placed in a NaI buffer to quench cellular fluorescence. The cells were then studied in a specially designed perfusion chamber at room temperature. Fluorescence of individual cells was measured using a Zeiss inverted microscope (excitation at 340 nm, emission at $>$ 410 nm; Zeiss, Thornwood, NY), a PTI imaging system (PTI, Monmouth Junction, NJ), and a Hamamatsu camera (Hamamatsu, Bridgewater, NJ). Baseline fluorescence was measured in isotonic NaI buffer, followed by perfusion with isotonic dequench buffer ($NaNO_3$ replaced NaI) to measure unregulated efflux, and then $NaNO_3$ buffer with 20 μM forskolin, 10 μM AA, or forskolin and AA as indicated. At the end of each experiment, cells were returned to the NaI buffer for requench (1,100 s). Increase in fluorescence above the basal (NaI quenched) level is shown (% increase F $>$ basal). The data are cumulative from three coverslips in each condition studied in a paired fashion on three separate days (n = 50 cells/curve). The bottom 10% of cells in all conditions (attributable to inadequate SPQ loading, cell detachment, etc.) were discarded and the data obtained from the top 90% of cells in each condition were analyzed as previously described (23, 25).

Real-Time RT-PCR to Quantify Ado Receptor Expression

A one-step Applied Biosystems PCR protocol was used to quantify CFTR mRNA transcripts using "*Assays on Demand*" Gene Expression Products, coupled with the ABI Prism 7,500 sequence detection system (Applied Biosystems, Foster City, CA). Briefly, total RNA was isolated using the Qiagen RNeasy mini kit according to manufacturer's instructions. To prevent possible DNA contamination, the samples were pretreated with RNase-free DNase (Qiagen, Valencia, CA) on columns following manufacturer's instructions. Sequence-specific primers and probes for human Ado receptors A1, A2A, A2B, and A3 AR, and 18S rRNA were purchased from Assays on Demand (ABI); (A1 AR—Assay ID: Hs00181231_m1, cytoband 1q32.1: boundary of exons 5/6; A2A AR—Assay ID: Hs00169123_m1, cytoband 22q11.23: boundary of exons 1/2; A2B AR—Assay ID Hs00386497_m1, cytoband 17p12-p11.2: boundary of exons 1/2; A3 AR—Assay ID: Hs00181232_m1, cytoband 1p21-p13: boundary of exons 1/2). TaqMan One Step PCR Master Mix Reagents Kit (ABI) was used for reverse transcription and PCR. The reaction volume was 25 μl including 12.5 μl of 2X Master Mix without UNG, 0.625 μl of 40X MultiScribe and RNase Inhibitor Mix, 1.25 μl of 20X target primer and probe, 5.625 μl of Nuclease-free water (Ambion, Austin, TX), and 5 μl of RNA sample. The reaction plates were covered with an optical cap and centrifuged briefly to remove bubbles. The thermocycler conditions were as follows: Stage 1: 48°C for 30 min; Stage 2: 95°C for 10 min; Stage 3: 95°C for 15 s; Stage 4: 60°C for 1 min; repeated ×40 cycles. All experiments were run in triplicate on two separate days. The absolute value of the slope of log input amount versus ΔCt was $>$ 0.1, implying that the efficiencies of AR and 18S rRNA amplification were not equal. Therefore, the relative quantification of transcript levels (AR compared with endogenous 18S rRNA) was performed using the standard curve method.

cAMP Measurements

Cellular cAMP was measured using an ELISA-based detection kit as previously described ((23, 26), Cayman Chemicals, Ann Arbor, MI). Briefly, cells grown on 60 mm dishes ($\sim 7 \times 10^6$ cells/dish) were stimulated with agonist for 10 min (37°C), and the cellular cAMP was extracted with ice cold ethanol. The supernatants were vacuum dried, resuspended in phosphate buffer, and the cAMP levels were quantified per manufacturer's directions.

Measurement of Prostaglandin and Leukotriene Synthesis

CFBE41o- and Calu-3 cell monolayers were grown as described above on 12-mm Costar inserts at an air–liquid interface, washed with PBS, and then stimulated with apical applied Ado or control media as described in the text. At the completion of the experiments, apical fluid ($\sim$ 200 μl) was removed and eicosanoids were measured using EIA kits (Cayman Chemicals, Ann Arbor, MI) following manufacturers' directions. Samples (agonist-stimulated and controls) were diluted 1:4 with MEM or EIA buffer after isolation to reduce the impact of endogenous inhibitors produced by airway cells.

Nucleotide Measurements

Studies in human subjects (sputum analysis, medical history review) were reviewed and approved by the UAB institutional Review Board and the Scientific Advisory Committee of the UAB General Clinical Research Center. After obtaining informed consent, spontaneously expectorated sputum samples were obtained from patients with CF with moderate lung disease and chronic infection with *Pseudomonas aeruginosa*. The amount of Ado in the extracts was measured using reverse-phase high-performance liquid chromatography (HPLC; Keystone Scientific, Inc., Bellefonte, PA) as described by Parker and coworkers (27). The amount of ATP in acid-soluble extracts of sputum samples were measured using Partisil strong anion exchange (SAX and reverse phase; Keystone Scientific) HPLC as described (23). Ado and ATP levels were determined by measuring its ultraviolet absorbance at 260 nm. Nucleotide concentrations were derived from HPLC standard curves.

Excised Membrane Patch Recordings

Macroscopic currents from Calu-3 cell membranes were recorded in the excised, inside-out configuration as previously described (28). Briefly, patch pipettes were pulled to tip resistances of 1.5–3 MΩ (when filled with pipette solution). CFTR channels were activated after patch excision by exposure of the cytoplasmic face of the patch to catalytic subunit of protein kinase A (PKA, 110 U/ml; Promega, Madison, WI) and MgATP (1.5 mM). CFTR currents were recorded in symmetrical solutions containing (in mM): 140 N-methyl-D-glucamine-Cl, 3 $MgCl_2$, 1 EGTA, and 10 TES (pH 7.3). Macroscopic currents were evoked using a ramp protocol from +80 to −80 mV with a 10-s time period and signals were filtered at 20 Hz. Data acquisition and analysis were performed using pCLAMP9.1 software (Axon Instruments, Foster City, CA). Experiments were performed at 21–23°C.

Statistical Analysis

For I_{sc}, EIA, Cl^- currents in excised membrane patches, cAMP measurements, nucleotide measurements, and RT- PCR studies, descriptive statistics (mean and SEM) and paired and unpaired *t* tests were performed using SigmaStat software (Jandel, CA). All statistical tests were two-sided and were performed at a 5% significance level (i.e., $\alpha = 0.05$).

RESULTS

Ado Receptor Expression in Human Airway Cell Monolayers

Previously we have detected A2B AR protein in human airway cells (Calu-3 and primary human bronchial epithelial cells), but we have not characterized relative expression of the four known Ado receptor subtypes in a variety of polarized human airway cells (19, 20, 24). For these studies, we used quantitative real-time PCR to identify Ado receptor expression in primary human airway epithelial cell monolayers (normal, non-CF), parental CFBE41o- (ΔF508/ΔF508 genotype), and Calu-3 cell monolayers. The CFBE41o- cells form polarized monolayers, and have recently been demonstrated to preserve many functions normally exhibited by intact human airway epithelia (23, 29, 30). Figure 1A shows mRNA levels of A1, A2A, A2B, and A3 ARs in all three cell types. A shared relative expression profile of A2B ARs > > A2A ARs > A1 ARs ∼ A3ARs was demonstrated all three human airway monolayer systems. Complimentary dose/response experiments with Ado in Calu-3 and CFBE41o- Wt monolayers are shown in Figure 1B. Maximal currents were produced after exposure to relatively high concentrations of Ado (∼ 10 μM), consistent with stimulation of the low-affinity A2B AR. Studies of A2B ARs often rely on demonstration of low Ado affinity for receptor function, as A2B receptor-specific agonists and antagonists do not clearly discriminate between activities of the A2B AR and other (higher affinity) adenosine receptor subtypes (16, 31, 32). Ado-stimulated currents have previously been shown to be sensitive to glybenclamide blockade in Calu-3 and CFBE41o- monolayers expressing wtCFTR (19, 20, 26), and no currents are produced by Ado/A2B AR agonists in parental CFBE41o- cells (ΔF508/ΔF508 genotype, nontransduced—confirming the wtCFTR-specificity of the results shown here [19, 23]). Currents stimulated by the A2 AR–selective agonist NECA were sensitive to PLA2 blockade with chlorpromazine (5 and 10 μM), while β2 AR–stimulated currents produced by albuterol were unaffected (Figure 2A, Calu-3 cells; Figure 2B, 16HBE cells). Coupled with our previous findings that Ado stimulates CFTR-dependent ion transport in human subjects (19), the results indicate that Ado regulates CFTR in both surface epithelial and serous airway cell types predominately through activation of A2B ARs.

Ado Levels in CF Sputum Samples

Since relatively high concentrations of Ado were necessary to fully activate CFTR-dependent Cl^- transport in wtCFTR-expressing airway cell monolayers, we investigated the concentrations of Ado nucleotides found in the lower airways of patients with CF. All patients ($n = 12$ samples over two separate days from six subjects with CF) had moderate lung disease based on FEV_1 (40–60%), and all provided sputum samples via spontaneous expectoration. Figure 3 summarizes the results of sputum nucleotide analysis by HPLC. Ado and ATP were readily detectable, with concentrations ranging from 2–65 μM for Ado. Since membrane-bound ectonucleotidases have previously been shown to rapidly dephosphorylate Ado nucleotides and produce Ado on airway surfaces (14, 24), the results here confirm that high levels of Ado and Ado precursors can be detected in the lower airways of patients with CF, at concentrations predicted to stimulate A2B ARs *in vivo*.

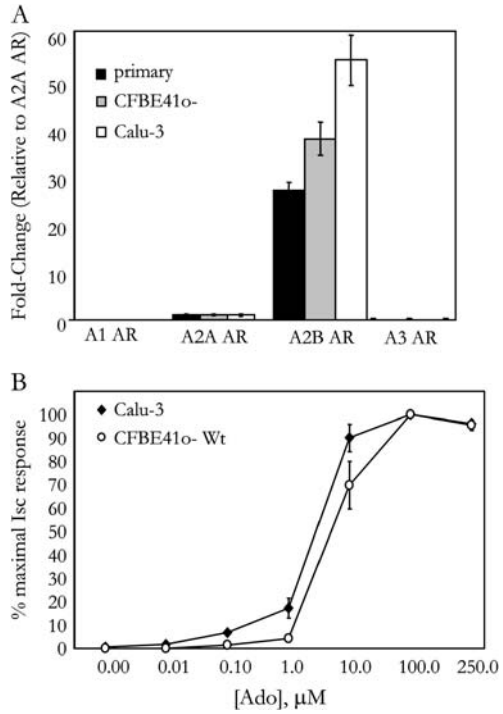

Figure 1. Expression of adenosine receptors in airway cell monolayers. (*A*) A2B ARs are detected at high levels relative to other Ado receptor subtypes in primary human airway cells, CFBE41o- Wt, and Calu-3 monolayers. Receptor RNA levels were normalized to endogenous 18S rRNA, and then ratioed to A2A AR transcript levels for comparison. (*B*) Dose–response studies in CFBE41o- Wt cells and Calu-3 cell monolayers demonstrate similar, full activation of CFTR-dependent Cl^- currents at ∼ 10 μM.

Ado Stimulates Prostaglandin Release from Airway Epithelial Cells

Previous work from our laboratory has implicated activation of cytoplasmic PLA2 after A2B AR stimulation, with release of free AA from the apical surface of Calu-3 cells (20, 21). Normally AA is a short-lived precursor to several metabolic products through the action of cyclooxygenase (COX-1 [constitutive] and COX-2 [inducible], which are then metabolized by specific prostaglandin synthases to TXA2 and multiple prostenoids) and 5-lipoxygenase (5-LO, giving rise to 5-HPETE, LTA4, B4, and the cysteinyl leukotrienes LTC4-E4). To examine whether COX and/or 5-LO activity was detectable after Ado stimulation, Calu-3 and CFBE41o- cell monolayers were stimulated overnight with Ado (10 μM), followed by sampling of the apical

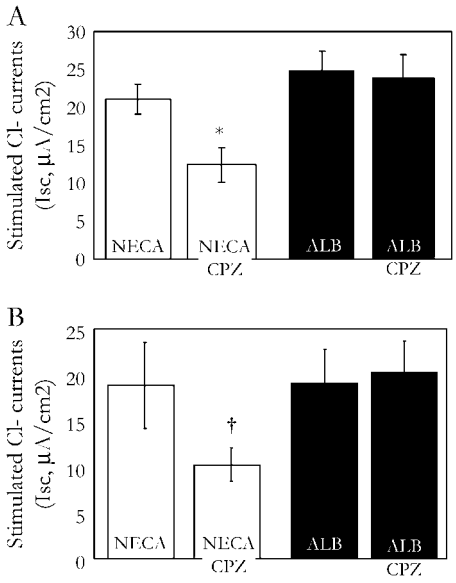

Figure 2. Adenosine stimulated currents are sensitive to PLA2 blockade. Stimulated currents after treatment with amiloride (100 μM) are shown (n = 6–14 filters in each condition, paired experiments). (*A*) Calu-3 cell monolayers were stimulated with NECA and ALB (0.1 μM) in the presence and absence of chlorpromazine (CPZ, 10 μM; *$P < 0.01$). Treatment with CPZ inhibited NECA- but not ALB-stimulated I_{sc}. 2B. 16HBE monolayers were stimulated with NECA and ALB (1 μM) in the presence and absence of chlorpromazine (CPZ, 5 μM; †$P < 0.05$). Treatment with CPZ inhibited NECA- but not ALB-stimulated I_{sc}.

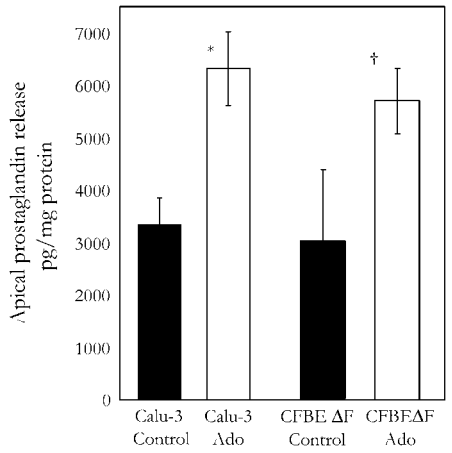

Figure 4. Prostanoid production by airway cell monolayers. Samples were obtained from the apical compartment after 16 h exposure to Ado (10 μM). Prostaglandin production in Calu-3 cells and CFBE41o-ΔF508 cells stimulated with Ado compared with controls. *$P < 0.005$ compared with controls; †$P < 0.05$ compared with controls.

compartment. As shown in Figure 4, prostenoids were readily detected in the apical compartment of Calu-3 cells and CFBE41o- stably transduced with ΔF508 CFTR, and production was enhanced after stimulation with Ado. Complimentary studies failed to detect 5-LO products after Ado stimulation (LTB4 and LTC4/LTD4, data not shown). The results confirm that Ado stimulates PLA2 activity in polarized human airway epithelia, leading to the release prostaglandin metabolites from the apical surface of human airway cell monolayers.

Ado Regulates CFTR through COX Metabolites

The findings from Figure 4 demonstrate relationships between Ado stimulation and prostenoid production in airway epithelia.

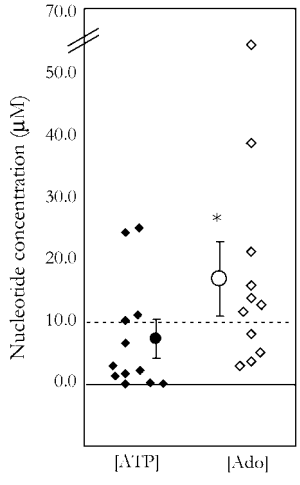

Figure 3. Adenosine nucleotide levels in CF sputum samples. ATP and Ado levels were measured in spontaneously expectorated sputum samples immediately after isolation. All patients (four male, two female, mean age 17 yr, range 10–33 yr) had moderate lung disease (FEV_1 range, 33–65% predicted), and all had positive sputum cultures for *P. aeruginosa.* Ado levels (mean 17.12 μM ± 6.10, *large open circles*) exceeded ATP levels (mean 7.40 μM ± 3.11, *large filled circles*; *$P < 0.05$). The *dotted line* at 10 μM represents the concentration of Ado that maximally activates I_{sc} (10 μM, Figure 1B).

To investigate whether COX metabolites contribute to CFTR regulation by Ado, CFBE41o- Wt cells were stimulated with NECA (10 μM) in the presence and absence of indomethacin (INDO, 20 μM), a potent inhibitor of COX activity. Control cells were stimulated with the PLA2 product AA, albuterol (ALB, a β2 AR agonist), or forskolin (a nonspecific stimulus of adenyl cyclase and cAMP production; Figures 5A–5D). The

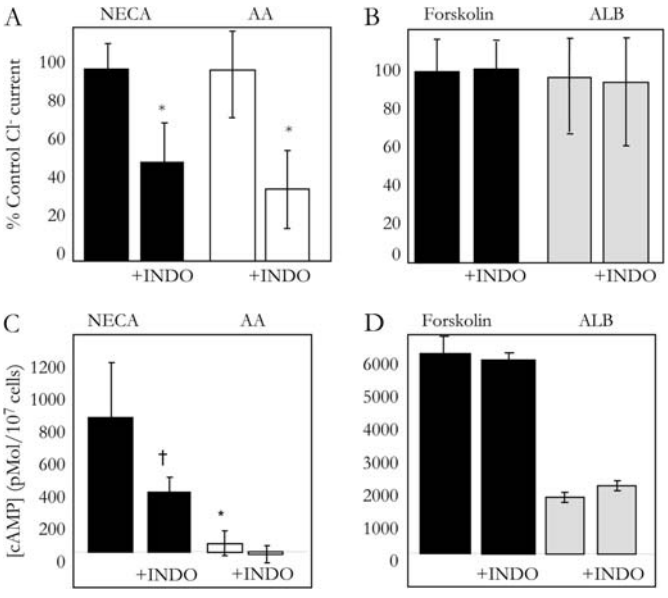

Figure 5. Effects of indomethacin (INDO, 20 μM) on Cl^- currents and cAMP production in CFBE41o- Wt cells after stimulation with NECA (A2 AR agonist), AA, ALB (albuterol, β_2 AR agonist), and forskolin. All agonists were studied at 10 μM. (*A*) NECA- and AA-stimulated I_{sc} are sensitive to INDO treatment (*$P < 0.01$ compared with controls). (*B*) Albuterol- and forskolin-stimulated I_{sc} are insensitive to INDO treatment. (*C*) NECA- and AA-stimulated cAMP are sensitive to INDO treatment (*$P < 0.01$ compared with controls; †$P < 0.002$ compared with controls). (*D*) Albuterol- and forskolin-stimulated cAMP are insensitive to INDO treatment.

results confirm that currents produced by Ado and AA were sensitive to INDO (Figure 5A), while inhibition of COX had no detectable effects on either ALB or forskolin-stimulated I_{sc} (Figure 5B). Complimentary studies with MK886 (an inhibitor of 5-LO–activating protein [FLAP], which normally shuttles AA from the cell membrane to 5-LO after liberation via cPLA2 [12, 13, 33]) demonstrated no effect on currents following NECA, ALB, or forskolin stimulation (data not shown). The findings confirm the specificity of the results for Ado receptors and AA, and point toward novel regulation of CFTR and Cl^- transport by prostenoids. Since CFTR is a cAMP/PKA regulated Cl^- channel, we also investigated the effects of the different agonists on cAMP production in CFBE41o- Wt cells in the presence and absence of COX inhibition. INDO was a potent inhibitor of cAMP produced by 10 μM NECA (Figure 5C), while forskolin and ALB-stimulation of cAMP were not affected by COX inhibition (Figure 5D). AA (10 μM) was a weak stimulus of cAMP production, which was also sensitive to COX blockade. Together, the findings indicate that both CFTR-dependent I_{sc} and cAMP production by NECA (and AA) stimulation are partly dependent on COX metabolism and prostenoids.

AA Activates CFTR in Airway Epithelial Monolayers

The results from Figure 5A demonstrate that AA stimulates Cl^- transport in polarized airway cells. To further define the nature of this stimulus, dose/response studies were performed in CFBE41o- Wt cells (Figures 6A and 6B), and in Calu-3 cell monolayers (Figure 6C). AA alone was a potent Cl^- secretagogue, producing maximal currents at ~10 μM in the CFBE41o- Wt and Calu-3 cells. AA failed to activate I_{sc} in CFBE41o- ΔF cells (stably transduced with ΔF508 CFTR, grown at 37°C), confirming the requirement for wtCFTR in the apical membrane to elicit Cl^- transport. In Figure 6C, Calu-3 cells were stimulated with apical AA at increasing doses in the presence and absence of basolateral membrane permeablization (with amphotericin, 500 μg/ml added to the basolateral compartment). Similar AA-stimulated currents were seen under both conditions, confirming that the effects of AA were localized to the apical cell membrane, and were not through stimulation of basolateral ion transport pathways (either K^+ channels or $Na^+/K^+/2Cl^-$ cotransporters). Treatment with H89 (20 μM) to block PKA activity effectively inhibited AA-activated currents, as would be expected for CFTR-dependent Cl^- transport. In Figure 7, CFBE41o- ΔF508 monolayers were studied after growth at 27°C for 48 h, a maneuver that has been previously shown to promote ΔF508 CFTR rescue to the cell surface (23, 30). Genistein dose–response experiments in the presence and absence of AA stimulation (10 μM) are shown. Similar to our previously published results with other receptor-based CFTR activators (23), AA had no clear synergistic effects on genistein-stimulated currents in temperature-corrected CFBE41o- ΔF monolayers.

To examine whether AA had direct or indirect effects (i.e., mediated by metabolic products) on CFTR-dependent Cl^- transport in intact monolayers, we stimulated CFBE41o- Wt and Calu-3 cell monolayers with AA (10 μM) in the presence and absence of INDO (to block COX activity) and MK886 (to block 5-LO activity). Figures 8A (CFBE41o- Wt cells) and 8B (Calu-3 cells) summarize the results. Inhibitors of COX and 5-LO each potently blocked AA-stimulated Cl^- currents. Complimentary cAMP measurements were performed in CFBE41o- Wt cells, and are shown in Figure 8C. Because AA was a less effective stimulus of cAMP relative to the other agonists studied at 10 μM (NECA, ALB, forskolin; Figures 5C and 5D), cells were stimulated with high (100 μM) concentrations of AA. Similar to the effects on I_{sc}, AA-stimulated cAMP production was sensitive to pretreatment with both INDO (20 μM) and MK886 (20 μM).

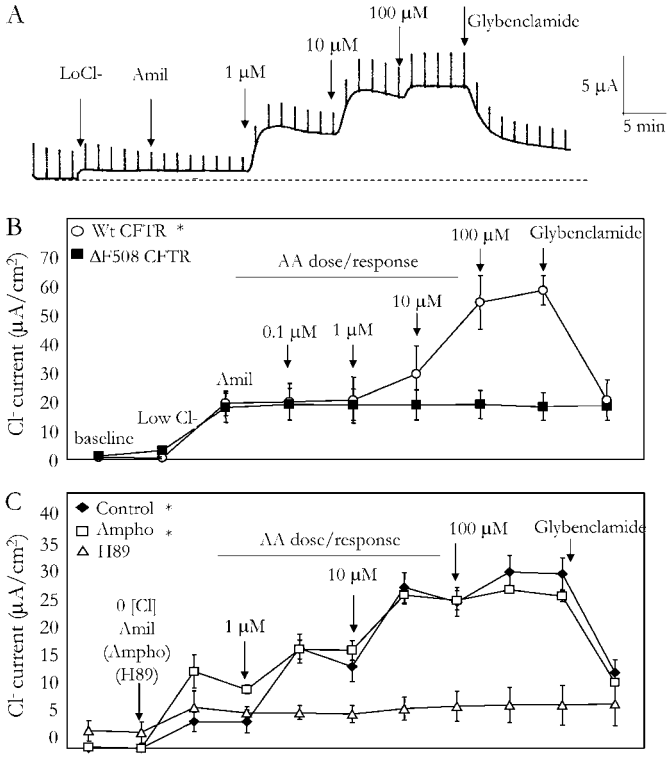

Figure 6. AA dose–response effects on Cl^- currents in CFBE41o- Wt, CFBE41o- ΔF508, and Calu-3 cell monolayers. (*A*) Representative voltage clamp tracing of dose–response experiment in CFBE41o- Wt cells. Currents were fully sensitive to glybenclamide blockade (200 μM, apical addition). (*B*) Summarized results from dose/response studies in CFBE41o- Wt and CFBE41o- ΔF508 monolayers (grown at 37°C). $*P <$ 0.001 compared with ΔF508-expressing cells following stimulation with 1, 10, and 100 μM AA. (*C*) Summarized results from AA dose/response studies (apical addition) in Calu-3 cells with and without basolateral membrane permeabilization (amphotericin, 500 μg/ml) or treatment with the PKA inhibitor H89 (20 μM). Similar dose–response relationships are seen with and without basolateral membrane permeabilization. AA-stimulated currents are completely blocked by H89 treatment. $*P <$ 0.001 compared with H89-treated cells after stimulation with 1, 10, and 100 μM AA.

To confirm that stimulation of CFTR-dependent ion transport by AA was indirect, cell type specific, and dependent on metabolic products of AA, HeLa cells stably expressing wtCFTR (HeLa Wt) were studied by SPQ (Figure 9A). In contrast to the results seen in the two airway cell lines, AA failed to stimulate halide efflux in HeLa Wt cells, and reduced halide efflux produced by forskolin. Figure 9B shows complimentary cAMP measurements in these cells. AA at concentrations of 10 and 100 μM failed to raise cAMP above control conditions. Together, the results of Figures 8 and 9 indicate that AA stimulates CFTR-dependent Cl^- transport in a cell type–specific manner, and requires metabolism by COX and/or 5-LO to activate CFTR-dependent I_{sc}.

AA Inhibits CFTR-Dependent Currents in Excised Membrane Macropatches

AA has previously been demonstrated to potently inhibit CFTR Cl^- currents through direct effects on channel function in heterologous expression systems (34, 35). As our findings pointed toward a potent CFTR-stimulatory effect in intact airway monolayers, we investigated the effects of AA on CFTR dependent Cl^- currents in excised membrane patches from Calu-3 cells.

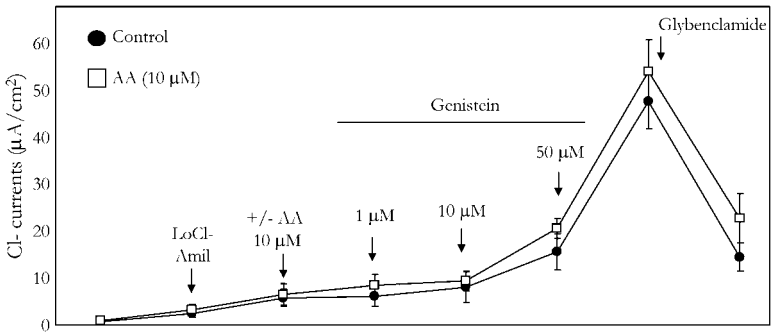

Figure 7. Effects of AA on genistein-stimulated I_{sc} in CFBE41o-ΔF508 cells grown at 27°C (48 h). Stimulation with AA (10 μM) had minimal effects on I_{sc} in airway cells treated to rescue ΔF508 CFTR to the cell surface. ΔF508 CFTR rescue was confirmed by subsequent activation of Cl^- currents by genistein. AA did not enhance genistein-stimulated Isc (1, 10, 50 μM).

Figure 10 summarizes the results. In contrast to the findings in monolayers, AA potently inhibited CFTR-dependent Cl^- currents in excised membrane patches after pre-activation with ATP (1.5 mM) and PKA (110 U/ml) (cytoplasmic [bath] addition of all agents). Figure 10A shows a representative experiment of AA inhibition of PKA/ATP-activated currents in an excised membrane patch, and Figure 10B summarizes the results from 10 experiments. These findings are in agreement with previously published results in which AA blocked CFTR currents in excised patches with a $K_i \sim 5$ μM (34). Cumulatively, the findings indicate a dual role for AA and metabolites in regulation of CFTR, activating CFTR-dependent Cl^- secretion in intact airway cell monolayers, but inhibiting CFTR currents after direct addition to activated CFTR channels.

DISCUSSION

The results of our studies identify important relationships between Ado, AA, prostenoid production, and CFTR regulation in airway epithelial monolayers. A2B ARs were the predominant adenosine receptor found in airway cells (including primary human bronchial cells, Figure 1A), and the dose–response relationships in both CFBE41o- Wt and Calu-3 cells were consistent with stimulation of CFTR through this low-affinity receptor (Figure 1B). The concentrations of Ado needed for full stimulation of CFTR and Cl^- secretion across airway monolayers were similar to those found in the sputum of patients with CF (Figure 3), and suggest that this agonist is available in relevant concentrations to stimulate A2B ARs within the airway environment *in vivo*. Coupled with our previous demonstration that 10 μM Ado activates Cl^- transport in the airways of normal human subjects (but not subjects with CF), the results provide compelling evidence to support the hypothesis that this pathway stimulates A2B ARs *in vivo*. The novel observation that Ado stimulates prostenoid release (Figure 4), and that both NECA and AA-activated currents and cAMP production were sensitive to COX inhibition (Figure 5), provide further support for the hypothesis that A2B ARs activate PLA2 and regulate CFTR through metabolic products of AA. These observations help to provide direct links between CFTR regulation and AA metabolism that are mediated through Ado and A2B ARs, and are among the first to identify signaling pathways that converge on CFTR regulation and the cPLA2/AA inflammatory signaling cascade.

Recent clinical studies have highlighted the importance of inflammation in CF, and agents that suppress AA metabolism through COX (e.g., ibuprofen) have been shown to be of significant benefit in treatment of CF lung disease (8, 36). While COX inhibitors might have other inhibitory effects on neutrophil function *in vivo* (37, 38), our findings suggest that they may reduce Ado-stimulated AA release and prostenoid production from the airway epithelium. The source of elevated Ado (and other Ado nucleotides) in CF sputum is uncertain, but is likely derived from a number of sources, including the airway epithelium itself, dead and dying inflammatory cells, and potentially lysed bacteria. Previous work by Tarran has highlighted the importance of shear stress (through the normal breathing cycle, and also as part of coughing) to release adenosine nucleotides onto the cell surface, where they stimulate P2Y2 receptors and "alternate" Cl^- transport as part of mucociliary clearance (3, 14, 15). It is also well known that various forms of stress (including hypoxia, infection, apoptosis, and cell death) can lead to egress of cellular nucleotides, and high bacterial counts within CF lower airway secretions (reported to reach concentrations as high as 10^9 cfu/g of sputum) could contribute to the high nucleotide content found in sputum (16, 39–41). Our findings extend those of Lazarowski and colleagues, where Ado levels in primary human airway epithelial monolayer cultures were lower than those measured in our studies (< 1 μM) (14). These differences suggest that the

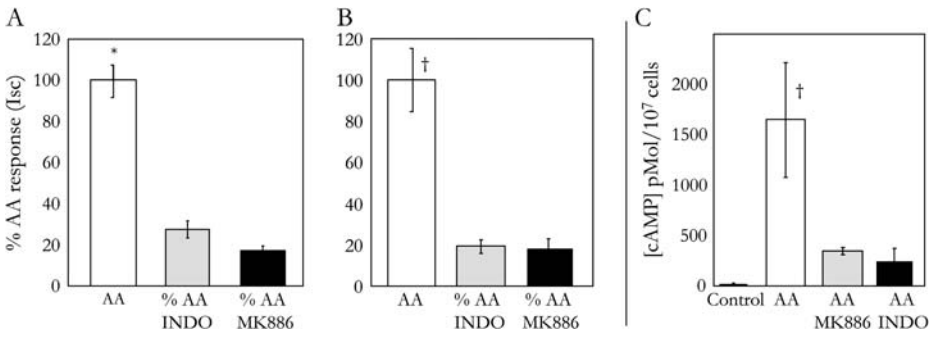

Figure 8. Effects of MK886 (20 μM) and INDO (20 μM) on AA-stimulated I_{sc} and cAMP production in airway cells. (*A*) INDO and MK886 each inhibit AA-stimulated I_{sc} (10 μM stimulation) in CFBE41o- Wt monolayers. $^*P < 0.001$ compared with AA alone condition. (*B*) INDO and MK886 each inhibit AA-stimulated I_{sc} (10 μM stimulation) in Calu-3 monolayers. $^\dagger P < 0.01$ compared with AA alone condition. (*C*) INDO and MK886 each inhibit AA-stimulated cAMP production (100 μM) in CFBE41o-Wt cells ($^\dagger P < 0.01$ compared with control, INDO, and MK886 conditions).

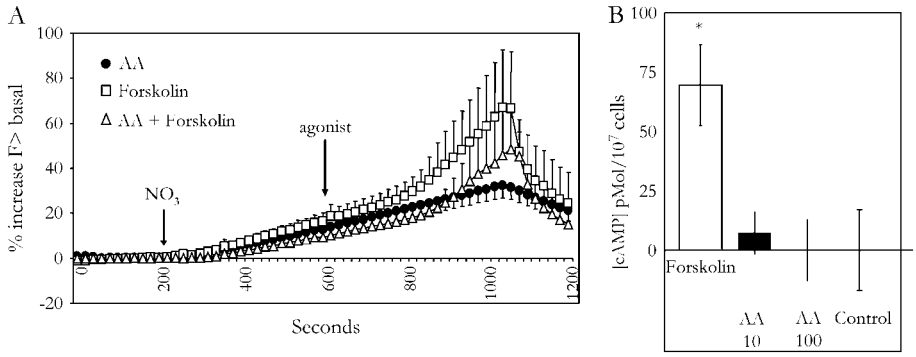

Figure 9. Effects of AA on halide efflux and cAMP production in HeLa cells stably transduced with wtCFTR (HeLa Wt cells). (*A*) SPQ studies in HeLa Wt cells. Forskolin stimulated halide efflux (10 μM, added at *arrow*). AA alone (10 μM) failed to stimulate halide efflux, while AA reduced forskolin-stimulated halide efflux. The *x* axis is time (s); the *y* axis is the increase in fluorescence above the basal "quenched" value (mean of fluorescence for all cells in NaI from 100–200 s). (*B*) cAMP production in HeLa Wt cells stimulated with forskolin (10 μM) or AA (10 and 100 μM). AA failed to stimulate AA production in HeLa cells. $*P < 0.02$ for forskolin compared with the other conditions.

elevation of Ado concentration (and related nucleotides) *in vivo* is likely part of CF pathology, and is not fully manifest in sterile cultures without the various cellular/infectious contributors found in the inflamed airway of patients with CF with established disease.

AA demonstrated dual regulation of CFTR function in airway cells. Exogenous AA activated CFTR-dependent currents in a cAMP- and PKA-dependent fashion in airway monolayers (Figures 5, 6, and 8), while direct application of AA to the internal (cytoplasmic) surface of excised membrane patches from Calu-3 cells inhibited CFTR-dependent currents in macropatches (Figure 10). ΔF508 CFTR was not activated by AA (Figure 8), and no synergy was seen when combined with increasing doses of genistein. AA was unable to activate wtCFTR or to stimulate cAMP production in HeLa cells (Figure 9), highlighting the indirect nature by which AA stimulates CFTR Cl^- currents in airway cell systems. The stimulatory effects of AA on CFTR have not previously been characterized, and were dependent on AA metabolism by COX and 5-LO (Figure 8). Both pathways produced cAMP after stimulation with high doses of AA, and we speculate that they may represent novel autocrine/paracrine regulatory pathways mediated by selective GPCRs of prostenoids and leukotrienes.

After conversion of AA to PGG2 and PGH2 by COX-1 or COX-2, prostaglandin synthases produce several prostenoids with diverse autocrine and paracrine functions, including TXA2, PGE2, PGF2α, PGI2, and PGD2. Each couples to one (or more) GPCRs, which have distinct cell types and tissues of expression. A total of nine prostenoid receptor subtypes have been identified, including TP (TXA2 agonist), DP and CRTH2 (PGD2 agonist), EP1–4 (PGE2 agonist), IP (PGI2 agonist), and FP (PGF2α agonist). Essentially all prostenoids and their receptors have been described to have both pro- and anti-inflammatory functions, which are dependent on their second messenger coupling and context of expression (for a recent review of the subject, *see* Ref. 12). Eicosanoids with well-described prominent airway/pulmonary effects (related primarily to asthma and airway function) include TXA2, PGD2, PGE2, and PGI2, and many of the accompanying receptors have been described to signal through G_s and cAMP. Recent studies suggest that EP2 and EP4 are expressed in Calu-3 cells, where they can activate CFTR, and an inverse regulatory relationship between IL-1β and PGE2 has recently been described in airway epithelia (42, 43). PGE2, PGF2α, and to a lesser extent 15-HETE have also been detected on the apical surface of porcine airway cell monolayers, which was augmented after stimulation with calcium-mobilizing agents and AA (44).

5-LO catalyzes the conversion of AA to 5-HPETE, which is then a substrate for 5-LO and FLAP to produce LTA4 (13, 33). Leukotriene A2 hydrolase converts LTA4 to the potent chemotactic agent LTB4, while leukotriene C4 synthase converts LTA4 to LTC4. Subsequent metabolism by g-glutamyl transpeptidase produces LTD4, which can then be metabolized by dipeptidases to LTE4. Cumulatively, LTC4, D4, and E4 are the cysteinyl leukotrienes that bind to LT-specific GPCRs and elicit diverse, proinflammatory cellular responses, including bronchoconstriction, mucous hypersecretion, granulocyte chemotaxis, and increased vascular permeability. Many of the LTs have been implicated in asthma pathogenesis, and potent inhibitors of receptors for the cysteinyl LTs are of significant benefit

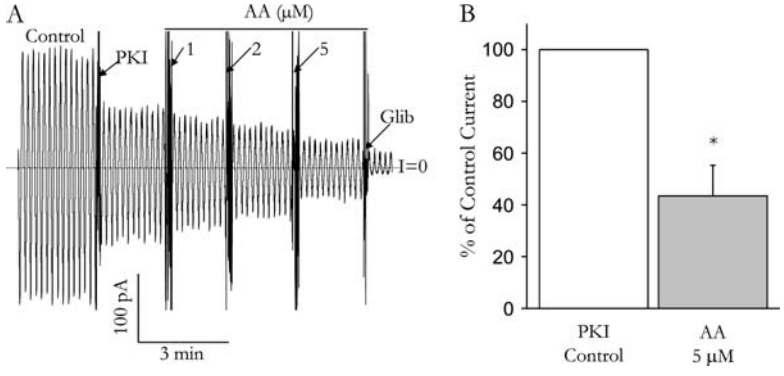

Figure 10. Effects of increasing concentrations of AA on Cl^- currents in excised membrane patches from Calu-3 cells. (*A*) A representative tracing from a membrane patch is shown. Macroscopic currents were evoked using a ramp protocol from +80 to −80 mV over a 10-s time period. Maximal currents (*left*) were recorded after addition of 110 U/ml PKA and 1.5 mM MgATP to the bath. Addition of PKI (1.4 U/ml, a specific inhibitor of PKA) reduced currents and confirmed their sensitivity to PKA blockade. Increasing concentrations of bath AA (1, 3, 5 μM) steadily reduced Cl^- currents. Glybenclamide (300 μM added to the bath) blocked essentially all residual Cl^- currents. (*B*) Summarized results of 10 separate experiments, demonstrating reduction of PKA/ATP stimulated Cl^- currents with 5 μM AA (mean residual current of 43.46% ± 3.73% compared with total currents, $*P < 0.01$ compared with currents before AA addition).

in the treatment of asthma and allergic-based lung diseases (13, 33). In the current study, we measured total Pg and LT production using EIA-based assays. Although LTs were not detected and Pg isoforms were not separated, future studies using more sensitive techniques may more clearly define the prostenoid species produced by chronic Ado stimulation, and define conditions that lead to LT production by Ado and/or stimulation of A2B ARs in airway epithelia.

Freedman and colleagues have recently demonstrated that AA levels are elevated in the membranes of CF affected tissues, and that restoration of AA:DHA ratios can reverse CF pathology in CF mice (9–11). The mechanisms of this damage are unknown, but it is tempting to speculate that Ado may play a role in these pathologic processes. Our results suggest that future studies investigating the role of Ado signaling in other CF-affected tissues, and the impact of elevated membrane AA content on Ado and A2B AR regulation of CFTR, prostenoid, and LT production, are warranted.

Finally, several important mechanisms of heightened airway inflammation have been described in CF, such as inherent alterations in IL-8:IL-10 release from airway epithelia, disordered CFTR regulation of nitric oxide synthase, and defects in lipoxin-mediated resolution of acute inflammation, among others (4–7, 45; for a recent review of CF pulmonary inflammation, *see* Ref. 46). How the findings reported here fit into these established disease mechanisms is unclear, but our results may represent an additional layer of inflammatory dysfunction in CF that also touches upon CFTR regulation.

In summary, the results presented here demonstrate tight regulatory links between Ado, CFTR, and production of AA metabolites in human airway cells. Evidence points toward mediation of these processes by A2B ARs, with coupling to both cAMP/PKA and AA/cPLA2 signaling pathways. Ado may serve an important "crossroad" function, providing plausible links between CFTR regulation and propagation of inflammation in the airway epithelium.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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