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Calcium-mediated, purinergic stimulation and polarized localization of calcium-sensitive adenylyl cyclase isoforms in human airway epithelia

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Abstract

Purinergic stimulation of human airway epithelia results in a prolonged increase in ciliary beat frequency that depends on calcium-mediated cAMP production (J. Physiol., 2002;538:633). Here, fully differentiated human airway epithelial cells in culture are shown to express calcium-stimulated transmembrane adenylyl cyclase (tmAC) isoforms (type 1, 3, and 8) by RT-PCR. Immunohistochemistry of tracheal sections and fully differentiated airway epithelial cell cultures revealed polarized expression of these tmACs, with type 1 and 8 localized to the apical membrane and thus at the position required for ciliary regulation. Real-time, ciliated-cell specific cAMP production by tmACs upon apical, purinergic stimulation with UTP was confirmed using fluorescent energy resonance transfer (FRET) between fluorescently tagged PKA subunits.

Keywords

cAMP production; adenylyl cyclase isoforms; ciliary beating; airway epithelium; polarized expression

Introduction

Regulation of mammalian airway ciliary beat frequency (CBF) is complex but is in part regulated by intracellular calcium and cAMP. Purinergic stimulation of airway epithelia via the P2Y receptor has been shown to be one of the strongest stimuli to increase CBF in a variety of mammalian species [1-11]. P2Y receptors couple to phospholipase C to produce IP_3 that releases calcium from internal stores. We have shown that mammalian CBF increases upon exclusive apical P2Y receptor activation with an initial calcium-coupled and a subsequent cAMP-dependent, prolonged ciliary beat activation [8]. The cAMP-mediated increase

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appeared to depend on calcium-mediated stimulation of a transmembrane adenylyl cyclase [8].

These results were interesting in several aspects. cAMP is not able to diffuse freely throughout the cell since its diffusion is restricted to microdomains by phosphodiesterases [e.g., 12,13], suggesting that transmembrane adenylyl cyclase (tmAC) isoforms responsible for the prolonged increase of CBF must be expressed at the apical membrane. In addition, the calcium-stimulated AC isoforms (type 1, 3, and 8) were thought to be mainly expressed in neural tissue [reviewed in 14] and expression of calcium-sensitive AC isoforms outside neural tissue has been shown only recently [e.g., 15,16]. Thus, the expression patterns of calcium-sensitive isoforms in the airway are unknown. Specifically, it remains unclear whether expression could occur in a polarized fashion. Furthermore, real-time measurement of cAMP production in single ciliated cells upon P2Y receptor stimulation has not been evaluated as such a technique has only been recently become available [17]. Therefore, we examined cAMP production in ciliated cells upon purinergic stimulation in real-time as well as the expression and localization of AC isoforms 1, 3, and 8 in human airway epithelia. Most experiments used cells cultured and re-differentiated at the air-liquid interface (ALI), which eliminates contaminating cells of non-epithelial, specifically neural origin, that could express calcium-sensitive tmACs.

Materials and Methods

Chemicals

Dulbecco's modified Eagle medium (DMEM), Ham's nutrient F-12, other media and Hank's balanced salt solution (HBSS) were purchased from Gibco, Life Technologies (Grand Island, NY). All other chemicals were purchased from Sigma (St. Louis, MO), unless stated otherwise.

Preparation of air liquid interface cultures of tracheal epithelium

Human airways were obtained form organ donors whose lungs were rejected for transplant. Consent for research was obtained by the Life Alliance Organ Recovery Agency of the University of Miami. All consents were IRB-approved and conformed to the Declaration of Helsinki. From these lungs, airway epithelial cells were isolated, de-differentiated through expansion and re-differentiation at an air-liquid interface (ALI) on 24-mm T-clear filters (Costar Corning, Corning, NY) as described [17-20].

Pseudo-typed lentivirus vectors and infection of airway epithelial cells

Third-generation, propagation-deficient HIV-pseudotyped lentiviruses encoding fluorescently tagged PKA subunits (RII-CFP and CAT-YFP) under control of the ciliated cell-specific *foxj1* promoter were used as described [17]. Undifferentiated cells were infected with these lentiviruses and were fully differentiated at the ALI. Upon differentiation, most ciliated cells expressed both fusion proteins and were used to measure changes in [cAMP] during purinergic stimulation with UTP.

RT-PCR analysis of mRNA expression of AC isoforms

To measure AC isoform expression in airway epithelial cells, RNA was isolated from cells cultured at the air-liquid interface, eliminating the possibility of contaminating cells that were not of epithelial origin. Total RNA was extracted using the RNeasy Protect Mini Kit (Qiagen, Valencia, CA). RNA samples were treated with DNase (DNase I Amplification Grade; Life Technologies) and precipitated with ethanol. Good quality of isolated RNA (28S to 18S rRNA ratio > 1.75) was confirmed using an RNA 6000 LabChip Kit and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). As a positive control, total RNA from human whole brain was purchased (Ambion). RT-PCR for AC1, AC3, and AC8 was achieved using gene-

specific RT and intron-spanning primers with 35 cycles of PCR. RT-PCR products were electrophoresed on ethidium bromide-stained 2% SeaKem agarose (BMA, Rockland, ME) gels. Control reactions were performed in the absence of RT. RT-PCR products were purified on silica spin columns (QIAquick PCR Purification Kit, Qiagen, Valencia, CA) and sequenced. Sequences were compared with the published cDNA sequences by PileUp (Wisconsin Package, GCG, Madison, WI). Table 1 lists the primer sequences.

Expression levels of AC1, AC3, and AC8 were compared using Quantitative Polymerase Chain Reaction (qPCR) and the ΔC_T method (compared to GAPDH). qPCR assays were performed using the BioRad iCycler iQ multicolor detection system (Hercules, CA) and SYBR green in real-time. Primer pairs were designed by the BeaconDesigner software program (Premier Biosoft, Palo Alto, CA).

Immunofluorescence

Immunofluorescence was performed on ALI cultures with AC1-, AC3-, or AC8-specific antibodies (all from Santa Cruz Biotechnology, Santa Cruz, CA). Cultures were fixed with 50% acetone/50% methanol for 2 min and incubated overnight at 4°C with anti-AC1 (Santa Cruz # sc-586), anti-AC3 (Santa Cruz, # sc-588), anti-AC8 (Santa Cruz, # sc-1967) antibodies, or antibodies pre-absorbed with an excess of the appropriate antigenic peptides (Santa Cruz, # sc-586p sc-588p, sc-1967p, sc-590p). Secondary antibodies were coupled to Alexa 555 (Molecular Probes, Eugene, OR). Axonemes were labeled using a monoclonal anti-acetylated α-tubulin antibody (Sigma Aldrich Saint Louis MO) and an Alexa 488-conjugated secondary anti-mouse IgG antibody (Molecular Probes, Eugene, OR). Cells were counterstained with DAPI before mounting in an aqueous anti-fading gel (Biomeda, Foster City, CA).

Fluorescent images were obtained using a Zeiss LSM-510 Confocal Laser Scanning Microscope. In addition, paraffin-embedded human tracheal sections were probed with anti-AC8 antibody. Enhanced fluorescence was achieved with the Tyramide Signal Amplification (TSA) kit (Molecular Probes, Eugene, OR), used according to the manufacturer's instruction.

Results

RT-PCR and mRNA comparison

RT-PCR using RNA isolated from ALI cultures was used with gene-specific primers to amplify fragments specific for AC1, AC3, and AC8. RT-PCR yielded DNA fragments of predicted sizes for each examined tmACs as compared to the positive control using human brain RNA (Figure 1). Sequencing of the amplimers revealed 100% nucleotide identity with published human sequences. The additional band at 556 bp seen in the AC3 PCR lane was an incompletely spliced form by sequencing. In the absence of RT, no PCR products were observed. Thus, AC1, AC3, and AC8 are expressed in airway epithelial cells re-differentiated at the ALI.

These data were confirmed with qPCR analysis for AC1, AC3, and AC8. These three tmACs were consistently expressed in ALI cultures at $1.1{\cdot}10^{-5} \pm 1.3{\cdot}10^{-6}$, $7.2{\cdot}10^{-4} \pm 7.5{\cdot}10^{-5}$, $5.5{\cdot}10^{-5} \pm 2.6{\cdot}10^{-6}$ fold the expression level of GAPDH (results from ALI cultures from 5 different lungs in duplicates).

Immunofluorescence localization

ALI cultures were probed with AC1, AC3, and AC8 antibodies and the mounted cultured were analyzed on a Zeiss laser confocal microscope with z-axis reconstruction (Figures 2-4). The AC1 antibody provided signal mainly at the apical pool of airway epithelial cells, specifically in ciliated cells (Figure 2). The same was true for AC8 antibodies (Figure 4). In contrast, staining with AC3 antibodies showed staining mainly on the basolateral aspect of airway

epithelial cells (Figure 3). Pre-absorption with the appropriate antigenic peptides eliminated staining, providing evidence for the specificity of the signals (Figure 5).

The location of AC8 was confirmed in tracheal sections and was also localized at the apical membrane (Figure 6).

cAMP production in human airway epithelial cells upon purinergic stimulation

Upon 100 μM UTP stimulation of cultured airway epithelial cells, cAMP increased as assessed by an increase in fluorescence resonance energy transfer (FRET) ratio (Figure 7). These cells expressed both tagged fusion proteins of PKA (CAT-YFP and RII-CFP) under the foxj1 promoter, which eliminates expression in non-ciliated cells [17]. The FRET ratio increase of $1.6{\cdot}10^{-3} \pm 0.13{\cdot}10^{-3}$ arbitrary units corresponds to the FRET increase seen when basolaterally permeabilized cells were exposed to 20 μM ATP [17]. To examine whether cAMP was produced by tmACs, cells were pretreated with 100 μM SQ22536, a general tmAC inhibitor. SQ22536 reduced the FRET ratio increase to $0.3{\cdot}10^{-3} \pm 0.1{\cdot}10^{-3}$ ($p < 0.05$ compared to UTP alone), confirming the decreased cAMP production in the presence of this inhibitor despite the presence of UTP [8]. DMSO, the solvent for SQ22536, did not influence the FRET response [17]. cAMP production occurs rapidly and transiently upon stimulation of cells with UTP. Thus, the prolonged, cAMP-dependent CBF increase in response to UTP is maintained by slow dephosphorylation events in the axonemes as reported by us before [17].

Discussion

cAMP has several important functions in the airway epithelium, including stimulating CBF [reviewed in 11,21,22-27]. In addition, cAMP is involved in the regulation of a major ion channel expressed at the apical surface, namely cystic fibrosis transmembrane conductance regulator or CFTR [e.g., 28,29]. cAMP is usually produced by G-protein coupled receptors activating tmACs. However, several isoforms of tmACs also produce cAMP upon increases in calcium concentration. Even though tmACs are reported to respond mainly to capacitative calcium influx rather than intracellular calcium release [reviewed in 14], we reported previously that capacitative calcium influx was not necessary to stimulate CBF in a cAMP-dependent manner upon purinergic stimulation in our human airway epithelial cells [8], a pathway supported by carbachol activation of AC8 in the absence of extracellular calcium in other cells [30]. Also consistent with these observations is the time course of cAMP production that occurs like the calcium increase after UTP stimulation, i.e. rapidly and transiently.

The data presented here for the first time show direct evidence of purinergic stimulation of cAMP-dependent activation of PKA using a FRET-based method adapted to normal airway ciliated cells [17]. The inhibition of cAMP production by SQ22536 also reveals that the calcium-dependent cAMP production in these cells upon purinergic stimulation [8] in fact depends on the activation of tmACs.

As already discussed above, there are at least three tmACs isoforms that are stimulated by calcium, namely AC1, AC3 and AC8. All of them were shown to be expressed in airway epithelial cells by RT-PCR, quantitative PCR and protein labeling, expanding the recent literature showing non-neuronal expression of these tmACs isoforms. Interestingly, the expression of these tmACs occurred in a polarized fashion, a fact that has not been appreciated before. These cyclases will therefore most likely fulfill polarized, i.e. apical- and basolateral-specific functions functions.

The protein localization of AC8 seen in ALI cultures was confirmed in tracheal sections. These results suggest that the ALI culture system is a good *in vitro* model for the *in vivo* tracheo-bronchial epithelium. We also examined mRNA expression data from a previous microarray

analysis that compared mRNA expression in ALI cultures to the native airway epithelium (in preparation). These experiments showed AC3 and AC8 were expressed at similar levels in surface epithelium and ALI cultures (< 2 fold differences). AC1 was not on the array.

In summary, human airway epithelial cells express calcium-sensitive tmACs isoforms in a polarized fashion. The expression of AC1 and AC8 at the apical membrane suggests that both are or at least one of them is involved in the purinergic regulation of ciliary beating.

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List of abbreviation

tmACs
transmembrane adenylyl cyclases

ALI
air-liquid interface

CBF
ciliary beat frequency

RT-PCR
reverse transcription polymerase chain reaction

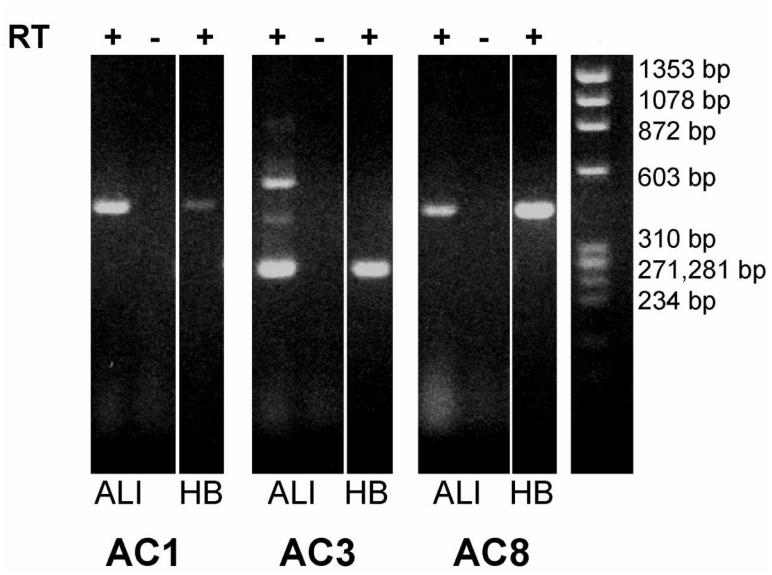

Figure 1.

mRNA expression of calcium-sensitive adenylyl cyclase isoforms in human airway epithelial cells. Adenylyl cyclase 1 (AC1), AC3, and AC8 mRNAs are expressed in human airway epithelial cells re-differentiated at the air-liquid interface (ALI). Control lanes without RT and RT-PCR from human brain (HB) RNA are shown. Sequencing of the amplimers revealed 100% nucleotide identity with published human sequences. The additional band at 556 bp seen in the AC3 PCR lane was an incompletely spliced form by sequencing.

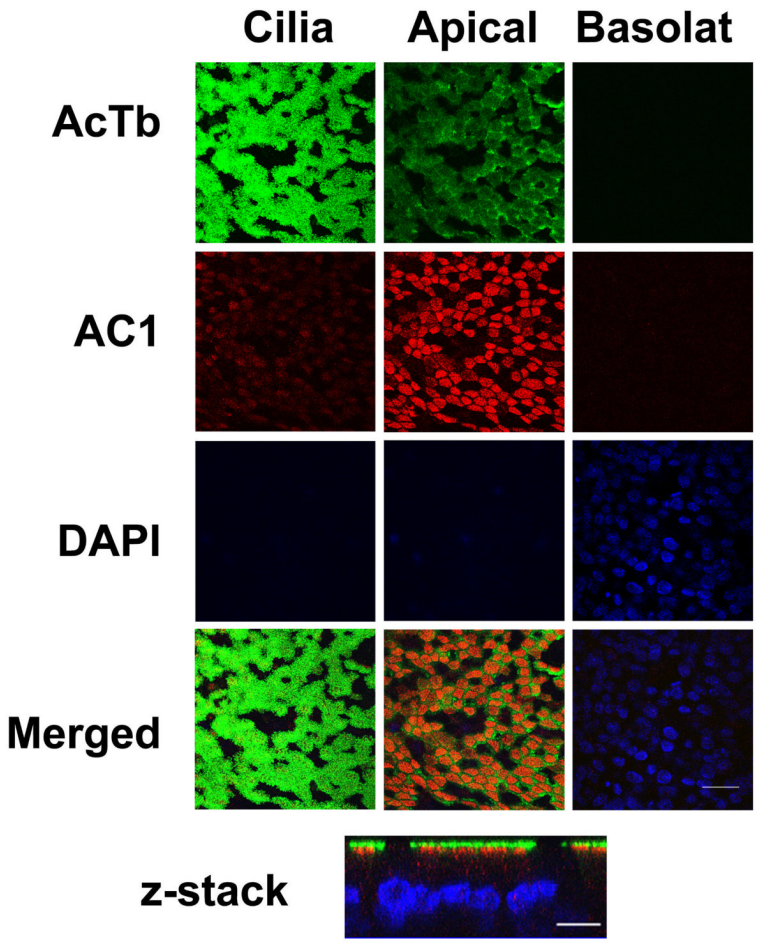

Figure 2.

AC1 is apically expressed in airway epithelial cells. Fully differentiated human ALI cultures were fixed and stained with antibodies against acetylated α-tubulin (AcTb; green) and against AC1 (secondary label Alexa 546 – red). Nuclei stained with DAPI (blue). Three levels obtained with confocal microscopy are shown (cilia, apical membrane below cilia, cellular or basolateral level) together with a reconstructed z-axis image (z stack). Bar is 20 μm in the xy plane and 10 μm in the z stack.

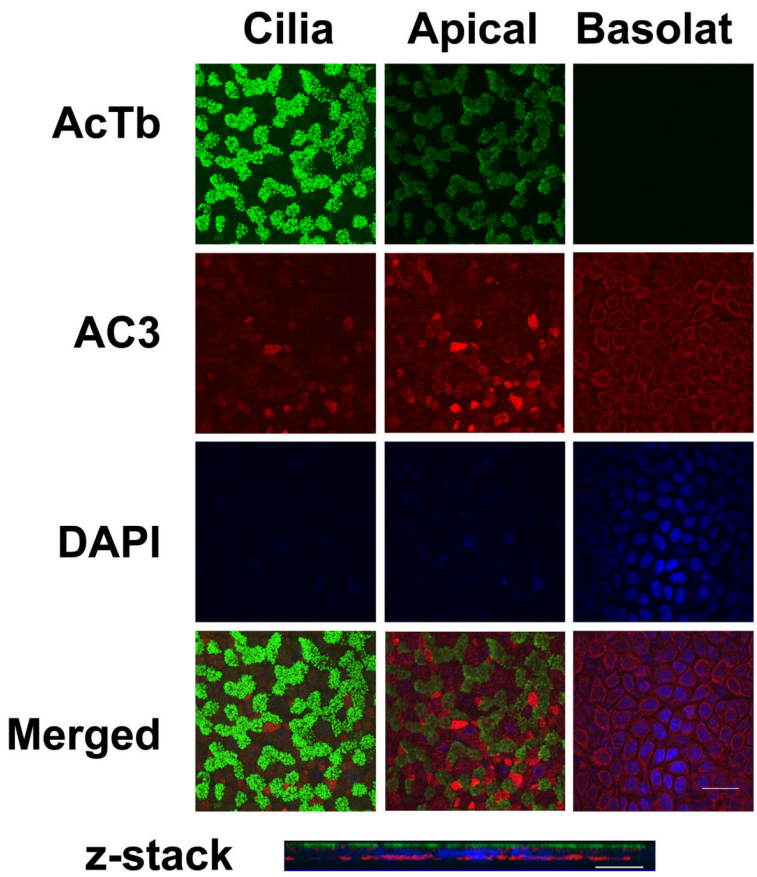

Figure 3.
AC3 is expressed at the basolateral side of ciliated cells. Apical expression occurs in non-ciliated cells. Labels as in figure 2, except that AC3 antibodies were used. Bar is 20 μm in the xy plane and 20 μm in the z stack.

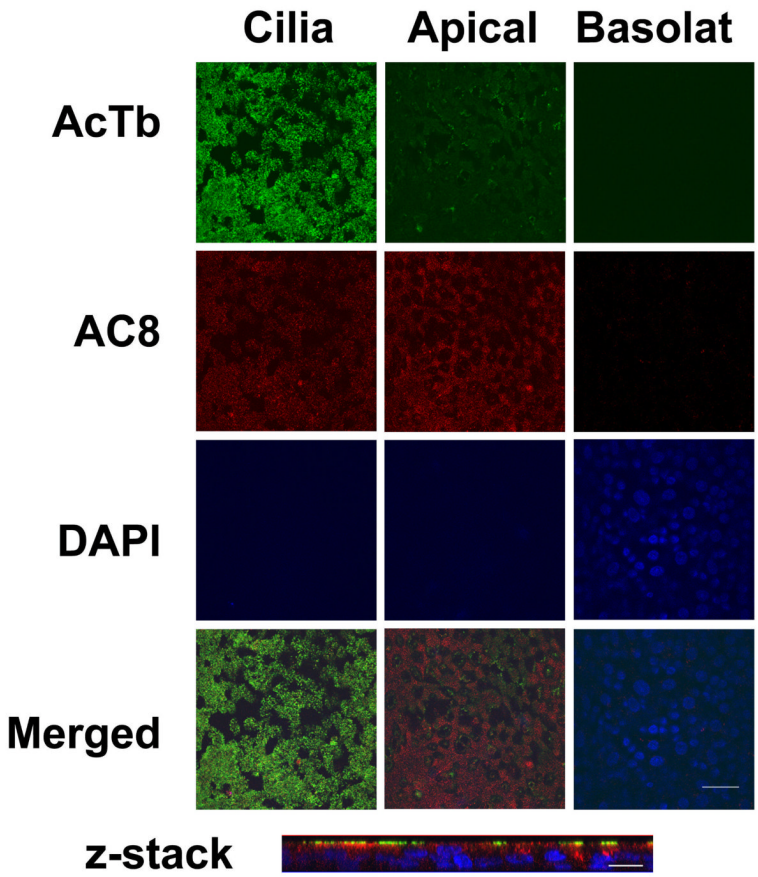

Figure 4.
AC8 is expressed at the apical membrane of ciliated and some non-ciliated cells. In addition, there was staining in an undetermined intracellular, apical compartment (z-stack). Labels as in figure 2, except that AC8 antibodies were used. Bar is 25 μm in the xy plane and 25 μm in the z stack.

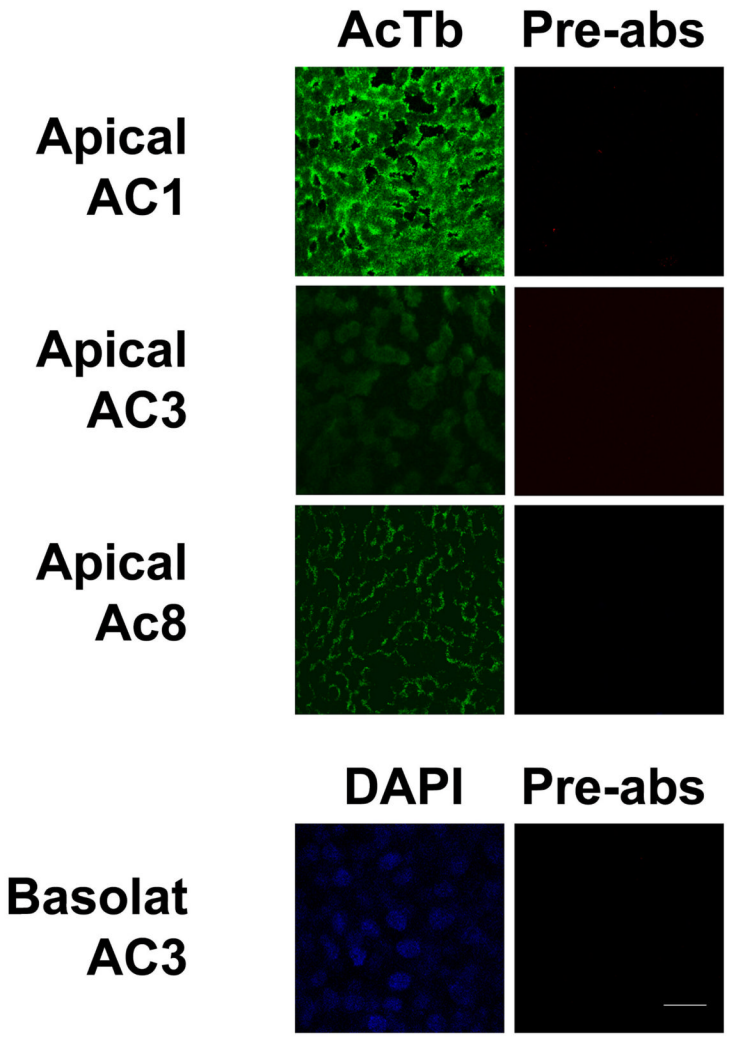

Figure 5.
Control cultures where antibodies were pre-absorbed with the respective antigenic peptide do not show any non-specific staining. Bar is 20 μm.

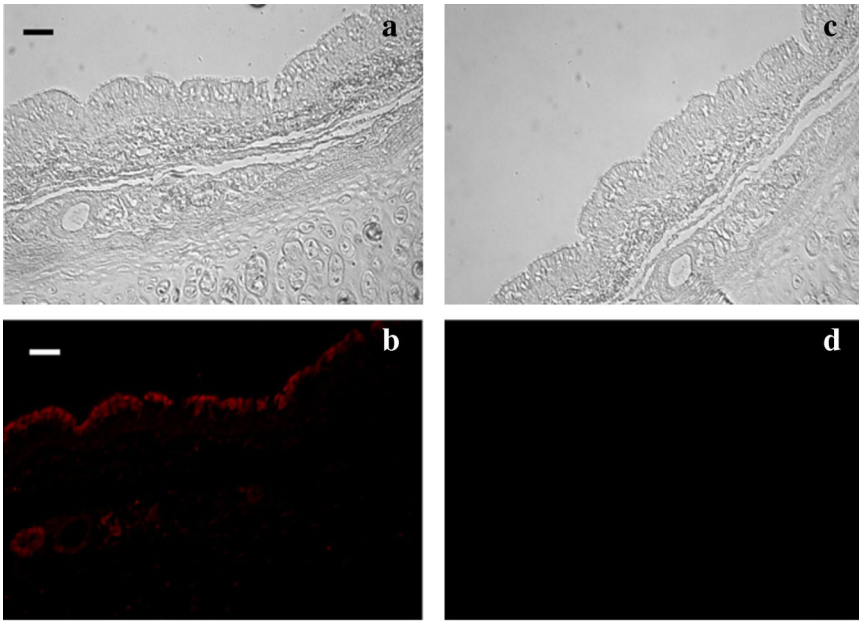

Figure 6.

AC8 staining in tracheal sections reveals expression at apex of superficial airway epithelial cells and submucosal gland cells. (a/c) Bright field illumination; (b/d) fluorescence of sections stained with AC8 antibody (a/b) and anti-AC8 antibody pre-absorbed with antigenic peptide (c/d). Bar is 20 μm.

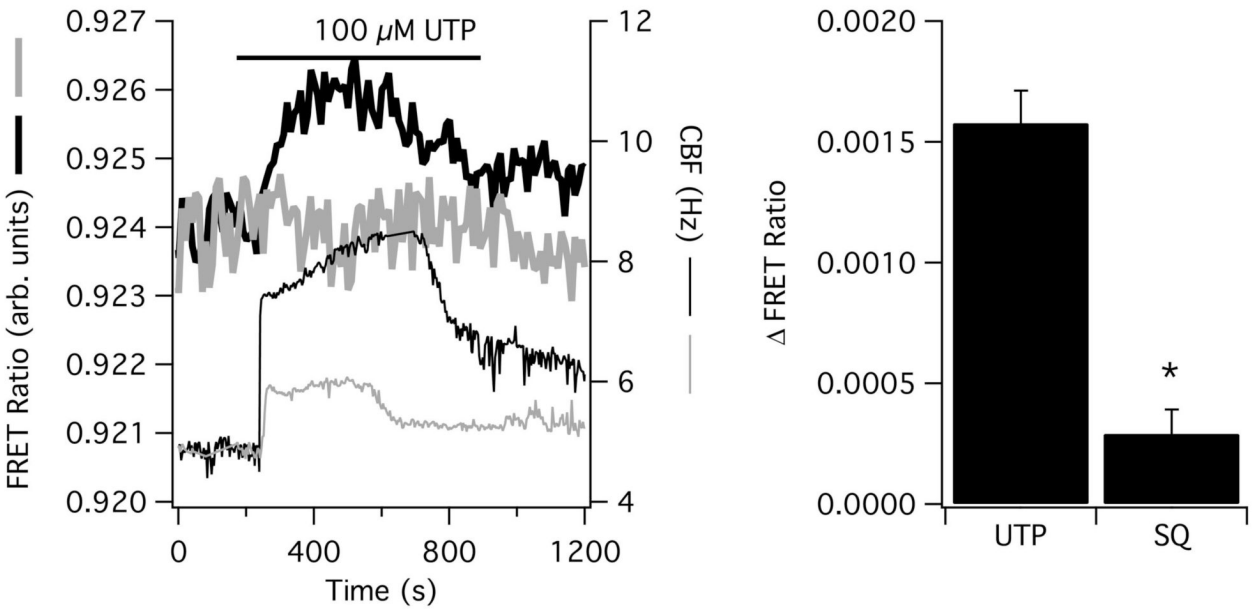

Figure 7.

Exposure of airway epithelial cells to the non-hydrolysable P2Y agonists UTP (100 μM) stimulates cAMP production in single ciliated airway epithelial cells. Cells were infected in a non-differentiated state with two lentiviruses encoding fluorescently tagged PKA subunits under the ciliated cell-specific fox-j1 promoter. FRET ratio was recorded while exciting the cells at the CFP excitation wavelength of 436 nm while simultaneously measuring emissions of both CFP and YFP. Left panel: Real-time, single cell traces of simultaneous measurements of FRET ratio (thick lines) between fluorescently tagged PKA subunits (indicative of cAMP concentrations) and ciliary beat frequency (CBF; thin lines) are shown. In black are the simultaneously recorded FRET and CBF traces of a cell stimulated in the absence and in gray a cell stimulated in the presence of 100 μM SQ22536, a transmembrane adenylyl cyclase inhibitor. SQ22536 inhibits calcium-mediated cAMP production. Right panel: summary data of the FRET ratio for $n \geq 29$ in each group. * $p < 0.05$.

Table 1

Primer sequences for RT-PCR

[[RT-PCR Primer]]			
[[Target]]	[[Forward]]	[[Reverse]]	[[Amplimer Size]]
[[AC1]]	[[5′-catgacctgcgaggacgat-3′]]	[[5′-acaggacagtgcgaatctgaa-3′]]	[[445 bp]]
[[AC3]]	[[5′-cacgggacccagcaat-3′]]	[[5′-gctctaaggccaccataggta-3′]]	[[263 bp]]
[[AC8]]	[[5′-cattggcagcacctacatgg-3′]]	[[5′-tgggttgggttggactcttc-3′]]	[[429 bp]]
[[qPCR Primers]]			[[]]
[[Target]]	[[Forward]]	[[Reverse]]	[[]]
[[AC1]]	[[5′-gccaatgccttgctcttc-3′]]	[[5′-tcagtcggtcctcaatgc-3′]]	[[]]
[[AC3]]	[[5′-gaacctctaccagacctac-3′]]	[[5′-caacaccagtccaattcc-3′]]	[[]]
[[AC8]]	[[5′-ccactctgacaggtctcc-3′]]	[[5′-gatgaggcgaaggtttgg-3′]]	[[]]