

Soluble Adenylyl Cyclase Is Localized to Cilia and Contributes to Ciliary Beat Frequency Regulation via Production of cAMP

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Ciliated airway epithelial cells are subject to sustained changes in intracellular $\text{CO}_2/\text{HCO}_3^-$ during exacerbations of airway diseases, but the role of $\text{CO}_2/\text{HCO}_3^-$ -sensitive soluble adenylyl cyclase (sAC) in ciliary beat regulation is unknown. We now show not only sAC expression in human airway epithelia (by RT-PCR, Western blotting, and immunofluorescence) but also its specific localization to the axoneme (Western blotting and immunofluorescence). Real time estimations of [cAMP] changes in ciliated cells, using FRET between fluorescently tagged PKA subunits (expressed under the *foxj1* promoter solely in ciliated cells), revealed $\text{CO}_2/\text{HCO}_3^-$ -mediated cAMP production. This cAMP production was specifically blocked by sAC inhibitors but not by transmembrane adenylyl cyclase (tmAC) inhibitors. In addition, this cAMP production stimulated ciliary beat frequency (CBF) independently of intracellular pH because PKA and sAC inhibitors were uniquely able to block $\text{CO}_2/\text{HCO}_3^-$ -mediated changes in CBF (while tmAC inhibitors had no effect). Thus, sAC is localized to motile airway cilia and it contributes to the regulation of human airway CBF. In addition, $\text{CO}_2/\text{HCO}_3^-$ increases indeed reversibly stimulate intracellular cAMP production by sAC in intact cells.

INTRODUCTION

A soluble form of adenylyl cyclase activity, enzymatically distinct from G protein–responsive transmembrane adenylyl cyclases (tmACs), was first described over 30 years ago in rat testis cytosol (Braun and Dods, 1975; Neer, 1978). However, its molecular identity remained unknown until 1999, when soluble adenylyl cyclase (sAC) was finally purified, cloned, and shown to be molecularly distinct from tmACs (Buck et al., 1999). Mammalian sAC activity is subject to different regulation than tmACs; sAC is insensitive to heterotrimeric G proteins and to forskolin (Buck et al., 1999), and its *in vitro* activity has been shown to be directly activated by $\text{CO}_2/\text{HCO}_3^-$ in a pH-independent manner (Chen et al., 2000; Steegborn et al., 2005b) and by Ca^{2+} , which synergizes with $[\text{HCO}_3^-]$ (Litvin et al., 2003).

The importance of sAC for axonemal beat regulation has been explored in sperm from mammals and echinoderms (Esposito et al., 2004; Hess et al., 2005; Nomura and Vacquier, 2006). Mammalian sAC has been identified in many tissues besides testis. Localization of sAC to different intracellular compartments within somatic cells (Zippin et al., 2003, 2004) suggested a model whereby intracellular cAMP-mediated signaling processes, which had been hard to explain when the only known sources of cAMP were membrane-localized tmACs, could be mediated by sAC-defined cAMP signaling microdomains (Bundey and Insel, 2004). Additionally, development of

pharmacological reagents selective for sAC relative to tmACs revealed that sAC is the source of cAMP in a number of physiological processes in somatic cells with tenuous historical links to this second messenger (Zippin et al., 2003; Han et al., 2005; Kamenetsky et al., 2006; Stessin et al., 2006; Wu et al., 2006).

Airway epithelial cells express motile cilia that are important for innate host defense by propelling mucus out of the airways. The regulation of flagellar beating (i.e., in sperm) differs from the regulation of cilia and thus information from sperm flagella may not be directly relevant to cilia. cAMP regulates airway ciliary beating through activation of PKA (Sanderson and Dirksen, 1989; Di Benedetto et al., 1991; Lansley et al., 1992; Salathe et al., 1993; Wyatt et al., 1998; Schmid et al., 2006). Thus, there is a shared requirement for an adenylyl cyclase, and because airway cells are likely exposed to sustained changes in intracellular $\text{CO}_2/\text{HCO}_3^-$ under stress during manifestations of airway diseases, sAC could be involved in regulating ciliary activity. Whether or not sAC even exists in ciliated airway epithelial cells, however, has not been explored. Here, we show that sAC is expressed in human airway epithelial cells, specifically localized to the axoneme and that sAC could contribute to airway ciliary beat frequency (CBF)

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Abbreviations used in this paper: ALI, air-liquid interface; CBF, ciliary beat frequency; FRET, fluorescence resonance energy transfer; ORF, open reading frame; sAC, soluble adenylyl cyclase; tmAC, transmembrane adenylyl cyclase.

TABLE I
Composition of Solutions

	#1	#2	#3	#4	#5
	APICAL	APICAL 25 mM HCO ₃ ⁻	BASOLAT	BASOLAT 25 mM HCO ₃ ⁻	BASOLAT no ATP
pH	7.4	7.4	7.2	7.2	7.2
NaCl	117	117	10	10	10
KCl	5.3	5.3	–	–	–
Na-gluconate	25	–	–	–	10
NaHCO ₃	–	25	–	–	–
KHCO ₃	–	–	–	25	–
K-gluconate	–	–	140	115	140
CaCl ₂	1.3	1.3	0.45 ^a	0.45 ^a	0.1
MgCl ₂	0.5	0.5	–	–	–
MgSO ₄	0.4	0.4	–	–	–
Na ₂ HPO ₄	0.3	0.3	–	–	–
KH ₂ PO ₄	0.4	0.4	–	–	–
Glucose	5.6	5.6	–	–	–
HEPES	20	20	20	20	20
Mg-ATP	–	–	10	10	–
CrP	–	–	10	10	–

Concentrations of solution components are given in mM. #3, #4, and #5 are solutions for permeabilized cells. pH adjustment with NaOH (#1), NaHCO₃ (#2), KOH (#3 and #5), and KHCO₃ (#4). Solutions containing HCO₃⁻ were equilibrated with 5%CO₂/95%O₂; all others with air.

^aApproximate concentration of free Ca²⁺ after chelation by ATP is 0.1 mM. CrP, creatine phosphate disodium.

regulation in conditions of changing intracellular CO₂/HCO₃⁻ via production of cAMP.

MATERIALS AND METHODS

Chemicals and Solutions

Unless stated otherwise, all materials were purchased from Sigma-Aldrich. The compositions of solutions used for apical and basolateral perfusion are shown in Table I.

Cell Cultures

Human airways were obtained from 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, dedifferentiated through expansion and redifferentiation at an air-liquid interface (ALI) on 24-mm T-clear filters (Costar Corning) as previously described (Bernacki et al., 1999; Nlend et al., 2002; Fragoso et al., 2004; Schmid et al., 2006).

Deciliation

Fully differentiated human airway epithelial cells grown at the ALI were washed with Dulbecco's modified phosphate-buffered saline. Then, 500 µl of deciliation buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM CaCl₂, 1 mM EDTA, 7 mM 2-mercaptoethanol, and 0.1% Triton X-100, see Hastie et al., 1986; Salathe et al., 1993) was applied to the apical side of the culture. The cultures were moved gently for a minute before the apical fluid was collected. Supernatant from low-speed centrifugation was recentrifuged at high speed and the resulting pellet containing axonemes was resuspended and stored at –20°C until use.

RNA Extraction, RT-PCR, and Sequencing

Total RNA was extracted from ALI-cultured human airway epithelial cells using the RNeasy Protect Mini Kit (QIAGEN),

subsequently 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 (Agilent Technologies) and an Agilent 2100 Bioanalyzer (University of Miami DNA Microarray Facility). RNA from testis, brain, liver, and kidney was purchased from Ambion. Total RNA (1 µg per sample) was used for first strand cDNA synthesis with Superscript II RT (Life Technologies) using oligo-dT₁₆ primers. For PCR amplification, oligonucleotide primers (forward: 5'-CTG AGC AGT TGG TGG AGA TCC TC-3'; reverse: 5'-CAG CCA GTC CTA TCT TGA CTC GG-3') were based on the published sequences of human sAC cDNA (GenBank/EMBL/DBJ accession no. NM_018417). PCR reactions (40 cycles of 30 s at 94°C, 45 s at 61°C, and 1 min at 72°C, final elongation of 10 min at 72°C) were performed using Taq DNA polymerase (Life Technologies). RT-PCR products were electrophoresed on ethidium bromide-stained 2% or 3% SeaKem agarose (BMA). RT-PCR products were purified on silica spin columns (QIAquick PCR Purification Kit; QIAGEN) and sequenced, followed by comparison with published cDNA sequences by PileUp (Wisconsin Package; GCG).

Cloning of cDNA Fragments of sAC RT-PCR Products

RT-PCR products were gel purified and ligated into pGEM-T Easy vector (Promega), expanded in DH5α *Escherichia coli* (Invitrogen), purified with the Wizard Plus SV Minipreps Purification System (Promega), restriction enzyme digested with EcoRI (New England BioLabs, Inc.), electrophoresed on 3% NuSieve GTG agarose gel (BMA), isolated, and sequenced.

Western Blot

ALI cultured human airway epithelial cells were lysed in the presence of protease inhibitors and briefly cleared from debris by centrifugation. Proteins were separated using SDS PAGE and electroblotted onto Immobilon P membranes (Millipore). Membranes were blocked with 10% nonfat dry milk and 0.05% Tween 20 and incubated with rabbit anti-sAC serum 1:10,000 (gift from

W. Geng, Southwestern Medical Center, Dallas, TX). This antibody was used since it works well for Western blotting. Chemiluminescence was used for detection on a ChemiDoc XRS system (BioRad Laboratories, Inc.). Membranes were stripped with Restore Western Blot Stripping Buffer (Pierce Biotechnology Inc.) and reprobed with rabbit anti- β -actin (1:100) (Sigma-Aldrich) or anti-acetylated tubulin (1:1,000) (Sigma-Aldrich). Human testis protein medley was purchased from BD Biosciences.

Pseudotyped 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 the ciliated cell-specific promoter *foxj1* were used as previously described (Schmid et al., 2006).

Immunohistochemistry

ALI cultures on filters were fixed in 50% acetone, 50% methanol, blocked with 1% BSA, and incubated overnight with the monoclonal mouse antibody R41 (1:100) made against a fusion protein corresponding to the 50-kD splice variant of human sAC (Zippin et al., 2003). This antibody was used because it works well for immunohistochemistry (while it does not work well for Western blotting). Secondary antibody (goat) was coupled to Alexa 555 (1:500; Invitrogen). After extensive washing with PBS, the cultures were reexposed 60 min to anti-acetylated α -tubulin antibodies (Sigma-Aldrich) using a secondary antibody coupled to Alexa 488 (both 1:500). DAPI (1:100; Invitrogen) was added to the last wash and incubated for 5 min before mounting in an aqueous anti-fading gel (Biomed). Even though both antibodies (R41 and anti-acetylated α -tubulin antibody) were monoclonal, specificity of secondary staining was confirmed by individual staining of acetylated α -tubulin in different cultures (see control in Fig. 3 A). Images were obtained at room temperature on a Carl Zeiss Micro-Imaging, Inc. LSM-510 confocal microscope using a Plan Apochromat 63 \times oil immersion objective with a numerical aperture of 1.4 and Carl Zeiss MicroImaging, Inc. acquisition software. Images were imported into Photoshop to assemble the figure (no deconvolution was performed and no gamma adjustments were made).

Human tracheal tissue sections were fixed with 4% formaldehyde, embedded, cut, deparaffinized, and permeabilized. Autofluorescence was reduced with NaBH₄ (5mg/ml) and nonspecific binding was blocked with 1% BSA for 90 min. Staining and visualization was otherwise done as described above for ALI-cultured cells, except that the images were obtained on a Nikon E600fn epifluorescent microscope using a Plan Fluor 10 \times objective with a numerical aperture of 0.3. Isee Imaging software from Inovision was used for image acquisition at room temperature. Images were imported into Photoshop to pseudocolor and to assemble the figure (no deconvolution was performed and no gamma adjustments were made).

Differentiated normal human airway epithelial cells grown on 0.4- μ m filters at the ALI and expressing the tagged PKA subunits were fixed with 4% formaldehyde and permeabilized with 0.1% Triton X-100 and 0.05% Tween 20 and nonspecific staining blocked with 2% BSA. Cilia were visualized with anti-acetylated tubulin antibody (1:500) using an Alexa 555-labeled goat anti-mouse antibody as secondary label (1:2,000). Images were obtained at room temperature on a Nikon E600fn epifluorescent microscope using a 63 \times water immersion objective with a numerical aperture of 1.0 and Metamorph software (Molecular Probes). Images were imported into Photoshop to assemble the figure (no deconvolution was performed and no gamma adjustments were made).

Measurement of CBF and FRET in Airway Epithelial Cells

ALI cultures of human airway epithelial cells were placed in a customized, closed chamber allowing independent perfusion of the

basolateral and apical compartment and mounted at room temperature on the stage of an upright Nikon E600fn microscope using a 63 \times water immersion objective with a numerical aperture of 1.0 (Schmid et al., 2006). If required, cells were permeabilized basolaterally as described before (Sutto et al., 2004).

CBF and FRET were estimated according to published methods (Salathe and Bookman, 1999; Schmid et al., 2006) using infrared differential interference contrast (IR-DC) video microscopy for CBF and simultaneous fluorescence. FRET ratios of CFP over YFP fluorescence were measured with Metafluor (Molecular Devices Corporation) as previously described (Schmid et al., 2006).

Statistics

Results were compared by one-way ANOVA and, if a significant difference was found, by the Tukey Kramer honestly significant difference test using JMP software (SAS). $P < 0.05$ was accepted as significant.

RESULTS

Expression of sAC in Human Airway Epithelial Cells: RT-PCR

To examine sAC mRNA expression in airway epithelial cells in comparison with other tissues, RT-PCR was performed with sAC-specific primers. The 5' primer is located in exon 3 and the 3' primer overlaps the splice junction between exons 5 and 6 (Fig. 1). Amplification of cDNA from human airway epithelial cells generated a major band of 280 bp with minor products of 243 and 292 bp. These products were also detected in other human tissues (Fig. 1 A). The RT-PCR products were cloned and sequenced and found to be alternative splice variants that differ at the location of the 3' splice junction of exons 4 and 5. The shortest, 243-bp amplicon (V1; Fig. 1, B and C) was identical to the human testicular sAC mRNA splice variant (GenBank/EMBL/DDBJ accession no. NM_018417). The 280-bp amplicon sequence is identical to a published sAC splice variant (Reed et al., 1999, 2002; Geng et al., 2005) having 37 extra nucleotides of the 3' end of the "intron" 4 sequence (GenBank/EMBL/DDBJ accession no. AF331033: V2; Fig. 1, B and C). The last, 292-bp amplicon was an unpublished splice variant containing 49 extra nucleotides derived from "intron" 4 (V3; Fig. 1, B and C).

The alternative splicing of these mRNAs affects the translational open reading frames (ORFs) of the mRNAs. The shortest, V1 form, of the mRNA has the largest ORF and encodes a sAC protein that has two catalytic domains in the N-terminal one third of the protein (Fig. 1 D). The longer amplicons, containing the sequences inserted between exons 4 and 5, alter the reading frame after amino acid 97 of the V1 ORF. These changes result in termination codons after an additional 10 (V2) and 14 (V3) amino acids. However, both of these insertions also introduce a new ATG translation initiation codon and four additional amino acids that are in frame with the C-terminal 1513 amino acid ORF of the V1 mRNA. Thus, the V2 and V3 encode identical

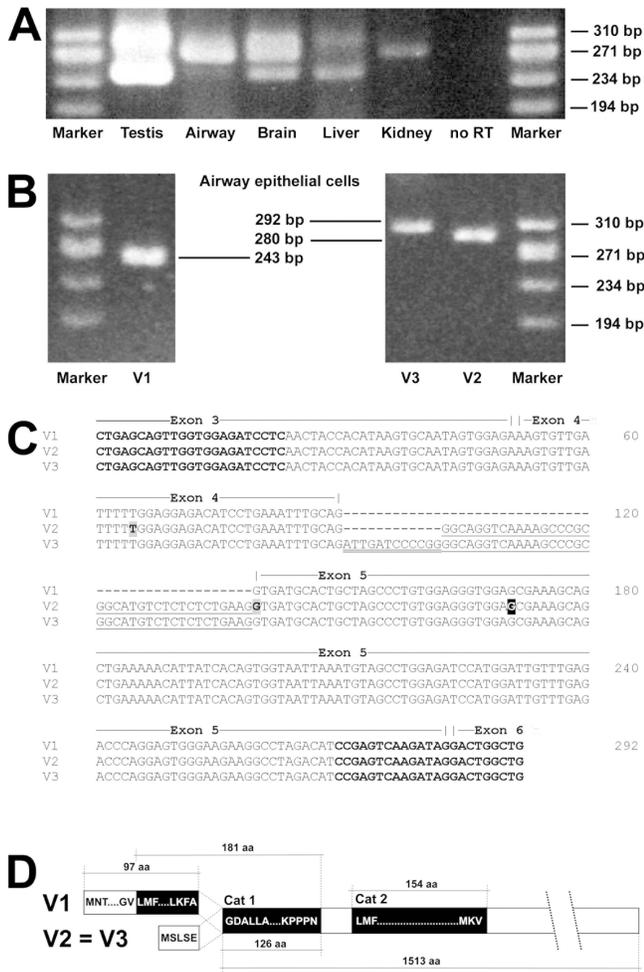


Figure 1. Expression of soluble adenylyl cyclase (sAC) mRNAs in human airway epithelial cells. (A) RT-PCR products from human airway epithelial cell RNA and other tissues after gel electrophoresis confirmed the presence of sAC cDNA of three different sizes. The source of the RNA is indicated at the bottom of each lane of the gel. No RT is the amplification of RNA from ALI cultures without reverse transcription. RNA was extracted from human airway epithelial cells grown at the ALI. Human testicular, brain, liver, and kidney RNA was purchased. (B) The RT-PCR products were cloned. Alternative splice variants with 243 (V1), 280 (V2), and 292 (V3) bp were found. (C) Sequencing of the vector inserts revealed a 243-bp fragment of human testicular sAC cDNA (V1) and two variants (V2 and V3). Bold-faced fragments represent the primers used for the PCR reaction; the extra 37 nucleotides in V2 and V3 are underlined and the insert of an additional 12 nucleotides in V3 is double underlined. Black and gray highlighted letters in V2 indicate variable bases in different clones. (D) Predicted protein structure of different splicing variants of sAC; V1 has the largest ORF and encodes a sAC protein that has two catalytic domains in the N-terminal one third of the protein. V2 and V3 initiate translation from a new ATG codon that is in frame with the C-terminal 1,513-amino acid ORF of the V1 mRNA. Thus, V2 and V3 encode identical proteins that differ from the V1 protein by deleting the first 97 amino acids and replacing them with five new amino acids. However, the remaining 1,513 amino acids are identical in V1, V2, and V3. The translated sequence of V1 has 42 amino acids at the N-terminal end (MNT...GV) in front of the first catalytic domain (Cat 1), which spans from LMF to KPPN and

proteins that differ from the V1 protein by deleting the first 97 amino acids and replacing them with five new amino acids. All three alternatively spliced isoforms, V1, V2, and V3, share 1,513 identical amino acids. Since these changes occur within the first of the two catalytic domains in the V1 protein, the V2 and V3 proteins encode proteins with only one complete catalytic domain (C2); the first catalytic domain (C1) found in the testicular isoform is missing its first 55 amino acids (Fig. 1 D). It is interesting to note that the V2 and V3 seem to represent the major forms of sAC expressed in airway epithelial cells by mRNA. In addition, another sAC variant missing the entire exon 5 has also been described (Geng et al., 2005); however, the reverse primer we used overlapped exon 5 and 6, therefore this particular sAC transcript would have escaped our notice.

Expression of sAC Proteins

To examine the expression and localization of sAC within airway epithelial cells, Western blots were performed with proteins prepared from whole cells, deciliated cells, isolated cilia/axonemes, and testis (positive control). To probe the Western blots, an antibody made against a peptide (SLSEGDALLA) corresponding to the extreme N terminus of human sAC proteins expressed from the V2 and V3 mRNAs (GDALLA is present in all proteins) was used that works well for this application (Geng et al., 2005). Immunoreactive bands were detected at ~50, 75, and 190 kD in whole airway epithelial cell and testis (control) extracts. In isolated cilia/axonemes, only the 50-kD band was detected, while this isoform seemed to be depleted in deciliated cell extracts (Fig. 2). For quality control of cilia/axonemes and deciliation, the same blots were stripped and reprobed with antibodies against acetylated α -tubulin and β -actin. Acetylated tubulin was greatly enriched in isolated cilia and in whole airway epithelial cells (Fig. 2) relative to deciliated cells, and less β -actin was only present in the cilia/axonemes recovered by centrifugation (Fig. 2). Thus, the method of deciliation resulted in a highly purified axonemal sample.

The sizes of immunopositive bands for sAC found in these blots were consistent with a previously described form of 50 kD and the full-length form of 187 kD (Chen et al., 2000) as well as a variant of ~80 kD (Geng et al., 2005). Interestingly, the 50-kD form seems to be localized preferentially to cilia/axonemes in airway epithelial cells.

Localization of sAC in Human Airway Epithelial Cells

To confirm the Western blot findings, we immunolocalized sAC in cultured human airway epithelial cells, fully

is 181-aa long. The N-terminal end of the protein of V2 and V3 starts with 5 aa (MSLSE) before a fraction of Cat 1 is expressed (GDALLA...KPPPN).

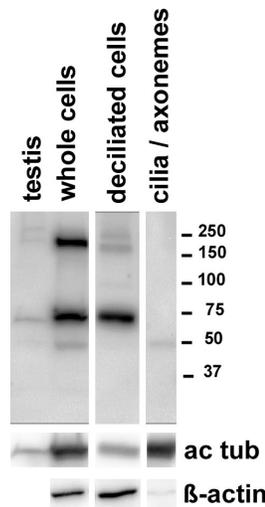


Figure 2. Protein expression of sAC in human airway epithelial cells. Western blot of human airway epithelial cell and human testes extract (positive control) with a polyclonal sAC antibody against the N-terminal sequence (SLSEGDALLA) of variants V2/3 (part of the sequence of the antigenic peptide, GDALLA, is present in all variants; see Fig. 1 D) shows three distinct, specific bands at \sim 190, 73, and 50 kD. Testis and whole human airway epithelial cells show all three bands. Cilia/axonemes contain only one band at \sim 50 kD, which is missing in deciliated cells, suggesting that this 50-kD form is localized to the axoneme. For quality control of axonemes and deciliation, blots were stripped and re-probed with antibodies against acetylated α -tubulin and β -actin. Ac tub, acetylated α -tubulin.

differentiated at the ALI, as well as in human trachea. We used a monoclonal mouse antibody against sAC (R41) recognizing an epitope in the second catalytic domain of human sAC (Zippin et al., 2003); we have found this epitope to be present in all known isoforms of sAC. In addition, this antibody works well for immunohistochemistry (but poorly for Western blotting). When used for immunofluorescence, cultured human airway epithelial cells revealed positive staining that colocalized with acetylated α -tubulin (indicative of localization to the axoneme) in addition to localization at the apical pole of airway epithelial cells (Fig. 3 A). The same results were found in tracheal sections (Fig. 3 B).

Ciliary Beat Frequency

To examine whether CBF is regulated by sAC, experiments were designed to change $[\text{CO}_2/\text{HCO}_3^-]$ in intact and basolaterally permeabilized airway epithelial cells. These experiments are complicated by the fact that changes in pH alone, independent of changes in $\text{CO}_2/\text{HCO}_3^-$, influence CBF directly, i.e., pH-induced changes are not mediated by PKA (Sutto et al., 2004).

To examine the effects of $\text{CO}_2/\text{HCO}_3^-$ on CBF, we first used intact, fully differentiated airway epithelial cells cultured at the ALI, mounted in closed chambers. These cultures were continuously perfused with HEPES-buffered Hanks' balanced salt solution (containing no

$\text{CO}_2/\text{HCO}_3^-$) and then temporarily exposed apically to 25 mM HCO_3^- solution bubbled with 5% CO_2 . Under these conditions, CO_2 rapidly diffuses into the cells, thereby acidifying them. In addition, this CO_2 -induced acidification is accompanied by a rise in intracellular $[\text{HCO}_3^-]$. Upon introduction of $\text{CO}_2/\text{HCO}_3^-$, CBF decreased by $19.3 \pm 1.2\%$ from a baseline of 6 ± 0.3 Hz in intact cells ($n = 20$; Fig. 4, A and C). This decrease is consistent with the acidification of these cells measured with BCECF (Sutto et al., 2004). After pretreatment and continuous perfusion of similar cells with KH7, a specific sAC inhibitor (Han et al., 2005; Stessin et al., 2006), CBF decreased by $27.7 \pm 1.5\%$ ($n = 20$, baseline not different from untreated cells) upon apical perfusion with $\text{CO}_2/\text{HCO}_3^-$. The CBF decrease observed in the presence of KH7 was thus significantly higher ($P < 0.05$) than the decrease seen in untreated cells (Fig. 4, B and C). This effect was not due to a toxic effect of KH7 on cilia since KH7 did not affect baseline CBF in control experiments and did not inhibit ATP-mediated increases in CBF, which are mediated by intracellular calcium. In HBSS, CBF baseline before KH7 addition was 5.3 ± 0.2 Hz and after 25 μM KH7 addition (10 min) 5.2 ± 0.2 Hz ($P > 0.05$). Addition of 10 μM ATP caused an increase of CBF to 6.7 ± 0.2 Hz ($n = 10$). In HCO_3^- -containing, CO_2 -bubbled buffer (Krebs Henseleit), baseline CBF before KH7 addition was 5.6 ± 0.3 Hz and after 25 μM KH7 addition (10 min) 5.4 ± 0.2 Hz ($P > 0.05$). Addition of 10 μM ATP caused an increase of CBF to 7.0 ± 0.3 Hz ($n = 8$). H89, another inhibitor used in these experiments used at 10 μM (see below), did also neither affect CBF baseline nor the CBF stimulation upon 10 μM ATP addition. In HBSS, CBF baseline before H89 addition was 4.6 ± 0.2 Hz and after 10 μM H89 addition (10 min) 4.6 ± 0.2 Hz ($P > 0.05$). Addition of 10 μM ATP caused an increase of CBF to 6.4 ± 0.3 Hz ($n = 16$). In HCO_3^- -containing, CO_2 -bubbled buffer (Krebs Henseleit), baseline CBF before H89 addition was 5.6 ± 0.3 Hz and after 10 μM H89 addition (10 min) 5.5 ± 0.3 Hz ($P > 0.05$). Addition of 10 μM ATP caused an increase of CBF to 7.4 ± 0.4 Hz ($n = 12$).

To examine the effect of increasing $[\text{CO}_2/\text{HCO}_3^-]_i$ on CBF further, normal human airway epithelial cell ALI cultures were basolaterally permeabilized with *Staphylococcus* α -toxin to eliminate influences of the basolateral membrane on the system and to control the permeable components of the cytoplasm (such as ATP and electrolytes) via basolateral solutions (Sutto et al., 2004; Schmid et al., 2006). When apically perfusing these cells with 25 mM $\text{HCO}_3^-/5\%$ CO_2 -buffered solutions (while perfusing basolaterally with a HEPES-buffered solution; Fig. 5 A), CBF decreased by $34.9 \pm 1.8\%$ from a baseline of 6.9 ± 0.2 Hz ($n = 44$; Fig. 5 C). This decrease was again likely due to acidification caused by CO_2 diffusion across the ciliary membrane (into cilia) at a higher rate compared with apical HCO_3^- influx

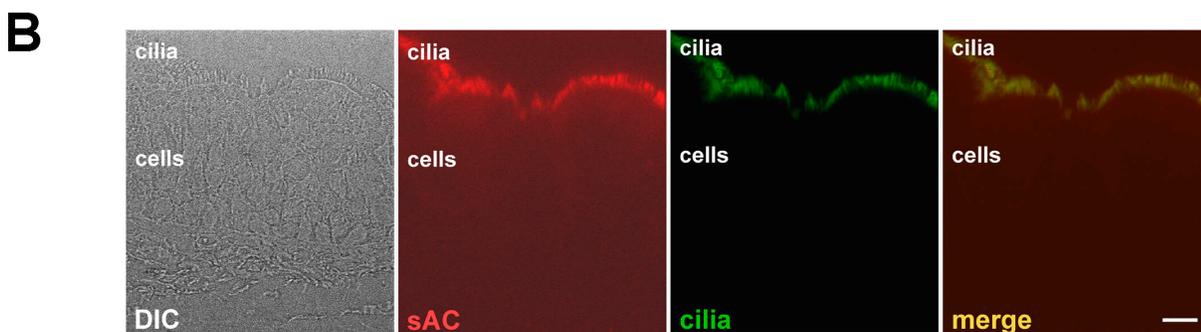
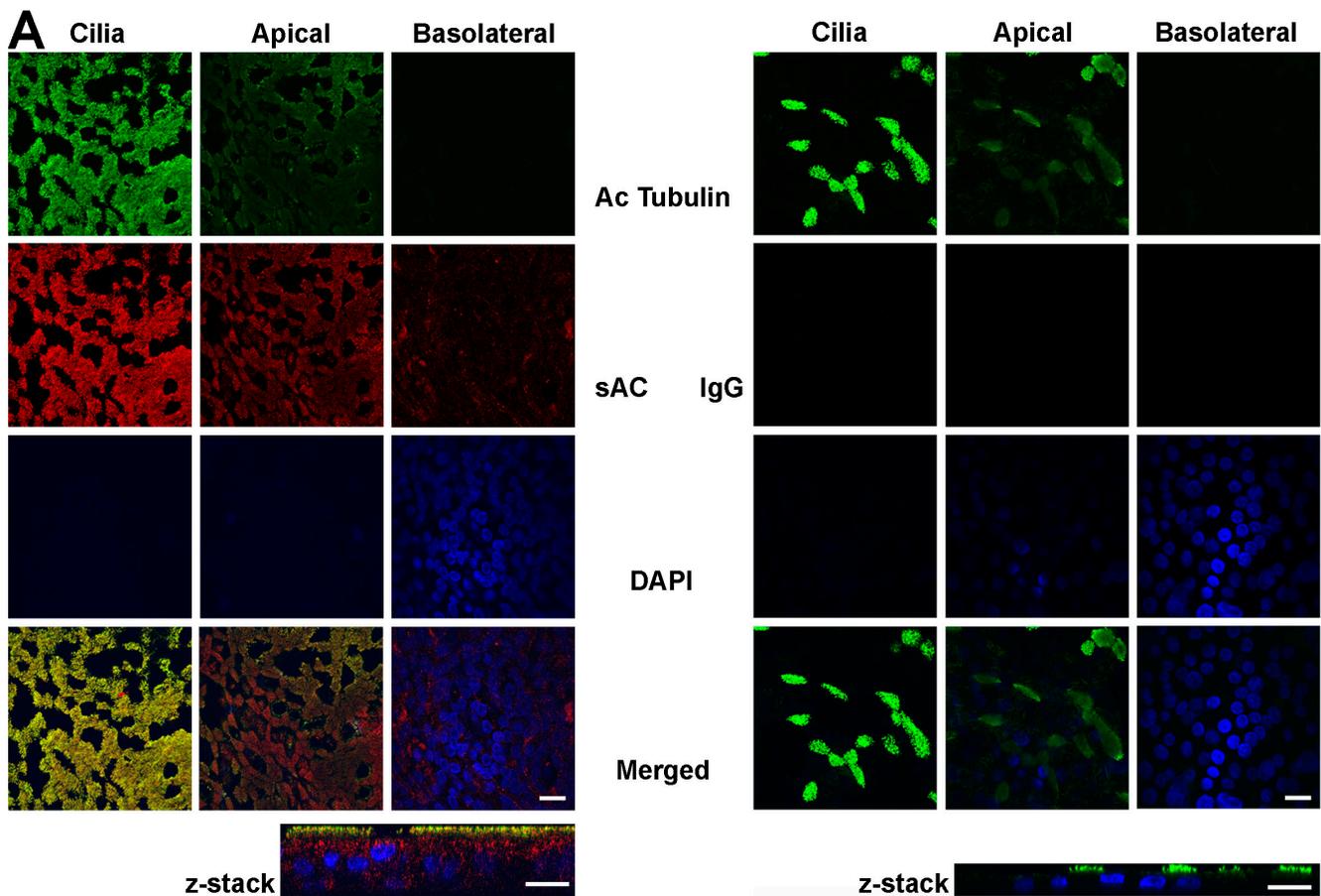


Figure 3. Immunostaining of human airway epithelial cells with monoclonal antibodies recognizing the truncated, 50 kD, sAC form. (A) Human airway epithelial cells grown and differentiated at the ALI were probed with a monoclonal mouse antibody against sAC (R41) (Zippin et al., 2003) and rabbit antibodies against acetylated α -tubulin (AC Tubulin) as indicated in the center. For visualization, secondary antibodies coupled to Alexa 555 for sAC and Alexa 488 for acetylated tubulin were used. These results show colocalization (yellow) of sAC (red) and acetylated tubulin (green) in cilia and in the apical compartment of the cells with little staining basally. The right panel shows a negative control using mouse IgG. Bars are 10 μ m. (B) Shown are the differential interference contrast (DIC) and immunofluorescent images of a tracheal section probed for sAC and acetylated tubulin using the same antibodies as in A. These images confirm data in ALI cultures, showing colocalization (yellow) of sAC (red) and acetylated tubulin (green) mainly in cilia. Bar, 10 μ m.

(i.e., the 10 mM HEPES in the basolateral perfusion solution provided insufficient buffering). Exchanging the basolateral solution with one containing 25 mM HCO_3^- /5% CO_2 (bilateral perfusion with HCO_3^- / CO_2) resulted in an increase in CBF of $18.6 \pm 3.7\%$ from a new baseline of 4.4 ± 0.2 Hz ($n = 44$), suggesting that the increase in $[\text{HCO}_3^-]_i$, now without the accompanying

acidification, was stimulating CBF (Fig. 5, A and D.). This HCO_3^- -induced increase in CBF could be mediated by cAMP generation following sAC activation; an alternative explanation would be that pH_i increased upon basolateral perfusion with HCO_3^- / CO_2 -buffered solutions. If the latter were the case, the HCO_3^- -induced change, would not be influenced by PKA or sAC inhibitors.

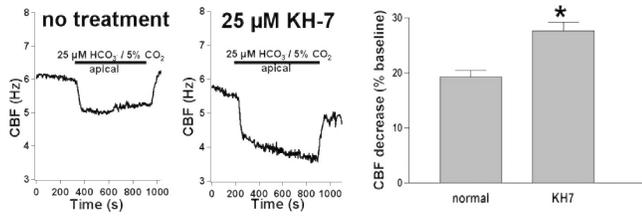


Figure 4. CBF response to changes in $\text{CO}_2/\text{HCO}_3^-$ in the presence or absence of the specific sAC inhibitor KH7. (Left and middle) Representative examples of CBF responses of fully differentiated, nonpermeabilized airway epithelial cells grown at the ALI to increases in $[\text{CO}_2/\text{HCO}_3^-]_i$ in the presence or absence of the specific sAC inhibitor KH7 (25 μM) are shown. CBF decreases by a larger degree when the cells are pretreated with KH7. (Right) Quantitative analysis confirms these results, suggesting that activation of sAC rescued the pH-mediated decrease upon acidification of the cytoplasm in control cells ($n = 20$ for both groups). *, $P < 0.05$.

To differentiate between these possibilities, PKA and sAC were inhibited by 10 μM H89 and 25 μM KH7, respectively. In permeabilized cells exposed to either inhibitor, CBF decreased upon sole apical perfusion with 25 mM $\text{HCO}_3^-/5\%$ CO_2 -buffered solutions by $40.5 \pm 1.8\%$ in the presence of H89 ($n = 36$) and by $41.6 \pm 1.3\%$ ($n = 22$) in the presence of KH7. For both inhibitors, the CBF decrease was significantly higher compared with noninhibited cells (see above; $P < 0.05$), while a control with DMSO did not change the CBF decrease significantly compared with normal cells ($P > 0.05$; unpublished data). In contrast, no significant increase from the new baselines was observed in the presence of either H89 or KH7 ($-0.3 \pm 2.2\%$ for $n = 36$ and $3.7 \pm 2.8\%$ for $n = 22$, respectively; $P < 0.05$ compared with control cells and $P > 0.05$ compared with new baseline) upon basolateral and apical perfusion with 25 mM $\text{HCO}_3^-/5\%$ CO_2 (Fig. 5, B and D). As noted above, these conditions increased CBF in control cells by $18.6 \pm 3.7\%$ from their baseline of 4.4 ± 0.2 Hz ($n = 44$). Again, DMSO had no effect ($P > 0.05$ compared with control cells).

These results suggested that $\text{CO}_2/\text{HCO}_3^-$ indeed regulated CBF in human airway epithelial cells and that the pathway of stimulation was $\text{CO}_2/\text{HCO}_3^- \rightarrow \text{sAC} \rightarrow \text{cAMP} \rightarrow \text{PKA} \rightarrow \text{CBF}$.

Real-Time Measurements of cAMP Production in Single Ciliated Airway Epithelial Cells

To test the pathway $\text{CO}_2/\text{HCO}_3^- \rightarrow \text{sAC} \rightarrow \text{cAMP}$ in ciliated cells further, we used our previously published fluorescent resonance energy transfer (FRET) method to measure real-time changes in cAMP in single ciliated cells (Schmid et al., 2006). Undifferentiated human airway epithelial cells were infected with two lentiviruses encoding the PKA fusion proteins RII-CFP and CAT-YFP, respectively, both driven by the ciliated cell-specific foxj1 promoter. Upon full differentiation of the cultures

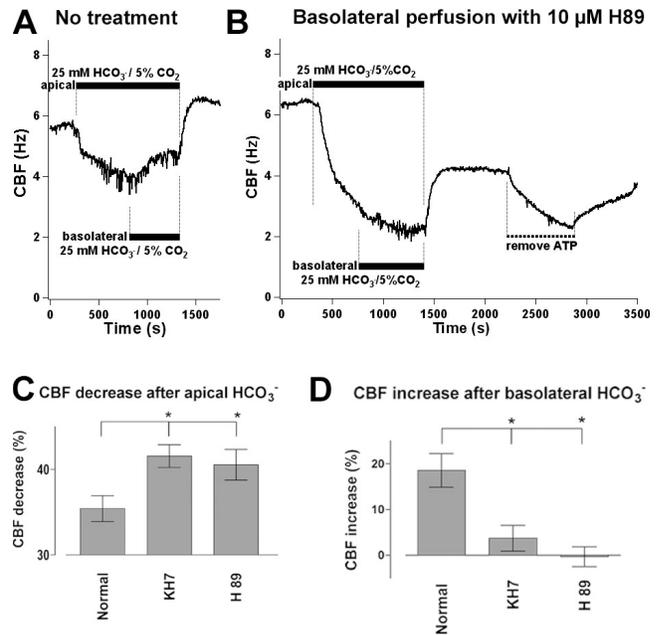


Figure 5. Ciliary beat frequency in basolaterally permeabilized cells in response to changing $\text{CO}_2/\text{HCO}_3^-$. (A) After obtaining a stable CBF baseline, basolaterally permeabilized cells were perfused apically with 25 mM $\text{HCO}_3^-/5\%$ CO_2 to saturate the cells with CO_2 . Consequently CBF decreased at least in part due to a pH_i decrease (Sutto et al., 2004). Subsequent basolateral perfusion with 25 mM $\text{HCO}_3^-/5\%$ CO_2 increases CBF. (B) After inhibiting PKA with 10 μM H89, CBF decreases significantly more in response to $\text{HCO}_3^-/\text{CO}_2$ than in the absence of the inhibitor. In addition, CBF does not increase upon basolateral perfusion with $\text{HCO}_3^-/\text{CO}_2$ in the presence of H89. At the end of the experiment, ATP was removed from the basolateral perfusate and the decrease in CBF proves successful permeabilization of the basolateral membrane. (C) Inhibition of PKA with 10 μM H89 and sAC with 25 μM KH7 causes significantly larger CBF decreases after apical perfusion with 25 mM $\text{HCO}_3^-/5\%$ CO_2 (each $n > 22$). *, $P < 0.05$, comparing groups indicated by brackets. (D) The increase of CBF after adding 25 mM $\text{HCO}_3^-/5\%$ CO_2 to the basolateral perfusate was also blocked by inhibiting PKA with 10 μM H89 and sAC with 25 μM KH7 (each $n > 22$). *, $P < 0.05$, comparing groups indicated by brackets.

at the ALI, ciliated cells expressing tagged PKA subunits (Fig. 6) were randomly chosen for FRET measurement (Fig. 7 A).

FRET ratio increased upon bilateral perfusion of basolaterally permeabilized cells with 25 mM $\text{HCO}_3^-/5\%$ CO_2 by 0.004 ± 0.0004 arbitrary units ($n = 12$), corresponding to an estimated [cAMP] increase of ~ 50 μM (Schmid et al., 2006). The FRET ratio increase upon perfusion with 25 mM $\text{HCO}_3^-/5\%$ CO_2 was unaffected by inclusion of 100 μM SQ22536, a selective inhibitor of tmAC but not sAC; the increase in the presence of 25 mM $\text{HCO}_3^-/5\%$ CO_2 plus 100 μM SQ22536 was also 0.0034 ± 0.0005 ($n = 16$), not significantly different compared with sole $\text{HCO}_3^-/\text{CO}_2$ perfusion ($P > 0.05$; Fig. 7, A and B). Inclusion of this concentration of SQ22536, however, was sufficient to block the FRET and

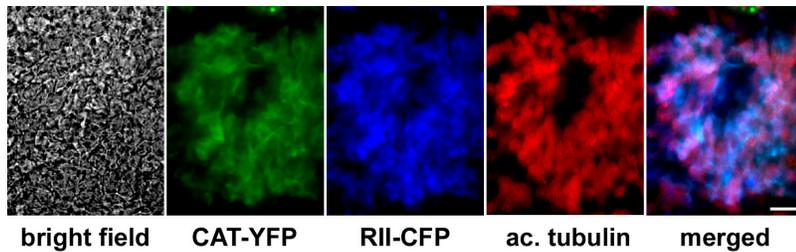


Figure 6. Expression of the PKA fusion proteins CAT-YFP and RII-CFP in fully differentiated, ciliated human airway epithelial cells. Undifferentiated cells were infected with lentiviruses encoding fluorescently tagged PKA fusion proteins under the ciliated cell-specific promoter *foxj1*. After full differentiation at the ALI, cells were fixed and cilia labeled with anti-acetylated α -tubulin (ac. tubulin) and Alexa Fluor-555-coupled secondary antibody. Nonciliated cells did not express the fusion proteins. Bar, 20 μ m.

CBF increase upon apical exposure of the cells with 20 μ M forskolin, a tmAC activator that does not influence sAC activity (unpublished data). Perfusion with 25 mM $\text{HCO}_3^-/5\%$ CO_2 containing 50 μ M of the sAC inhibitor 2-hydroxy-17 β -estradiol (Steegborn et al., 2005a) increased the FRET ratio by only 0.00024 ± 0.00018 ($n = 29$), significantly less compared with sole $\text{HCO}_3^-/\text{CO}_2$ perfusion ($P < 0.05$; Fig. 7 B). In contrast, perfusion with 25 mM $\text{HCO}_3^-/5\%$ CO_2 containing 50 μ M of estrone that does not inhibit sAC increased the FRET ratio by 0.0035 ± 0.00024 ($n = 21$), a value not significantly different compared with $\text{HCO}_3^-/\text{CO}_2$ perfusion alone. Perfusion with 25 mM $\text{HCO}_3^-/5\%$ CO_2 containing 25 μ M of the sAC-specific inhibitor KH7 increased FRET ratio by only 0.0003 ± 0.00014 ($n = 21$), again significantly less than perfusion with $\text{HCO}_3^-/\text{CO}_2$ alone ($P < 0.05$; Fig. 7 B). Inhibiting dissociation of the reporter construct (labeled PKA) with 400 μ M 8-Br-cAMPS (a PKA inhibitor) also eliminated FRET ratio increases upon bilateral $\text{HCO}_3^-/\text{CO}_2$ perfusion (change by only 0.00009 ± 0.00002 ; $n = 28$; $P < 0.05$ to $\text{HCO}_3^-/\text{CO}_2$ and $P > 0.05$ to baseline), indicating that the changes in FRET ratio truly reflected cAMP-mediated dissociation of the tagged PKA and not a nonspecific influence on fluorescence (Fig. 7, A and B). Thus, these data show that $\text{HCO}_3^-/\text{CO}_2$ in fact stimulates production of cAMP, which stimulates CBF, and that cAMP is produced in a manner consistent with sAC, but not tmAC, activation.

Since YFP fluorescence can decrease upon acidification (Griesbeck et al., 2001), possibly mimicking an increase in FRET ratio (even though the data using 8-Br-cAMPS argued against such a possibility), we compared bilateral $\text{HCO}_3^-/\text{CO}_2$ perfusion with bilateral exposure of permeabilized cells to solutions with different pH (Table I). Previous experiments have shown that the decrease in pH_i upon perfusion with 25 mM $\text{HCO}_3^-/5\%$ CO_2 are in the range of 0.5 pH units (Sutto et al., 2004). Thus, solutions with pH of 6.5 and 8 were chosen for comparison. Bilateral perfusion of basolaterally permeabilized cells with solutions of pH 6.5 increased apparent FRET ratio by only 0.0016 ± 0.0004 ($n = 12$), significantly less than the value seen with 25 mM $\text{HCO}_3^-/5\%$ CO_2 in date and culture-matched controls (0.004 ± 0.0004 , $n = 12$; $P < 0.05$). pH 8.0 had a negligible effect on the FRET ratio (change of $0.0004 \pm$

0.00051 , $n = 12$; $P > 0.05$ to baseline; Fig. 7 C). Thus, changes in YFP fluorescence due solely to acidification are not responsible for the observed changes in the FRET ratio upon 25 mM $\text{HCO}_3^-/5\%$ CO_2 perfusion, we can conclude that cAMP is produced in these cells due to sAC activation.

DISCUSSION

The discovery of sAC expression in cells other than sperm and the ability of sAC to produce cAMP in vitro upon increases in $\text{CO}_2/\text{HCO}_3^-$ has shed light into the question of how cAMP could be produced within cytoplasmic microdomains that are away from the plasma membrane since these microdomains are surrounded by phosphodiesterases that restrict free diffusion of cAMP throughout the cell (e.g., Mongillo et al., 2006; Terrin et al., 2006). sAC has now been implicated to regulate many cellular functions and is strategically localized to areas with putative changes in $\text{CO}_2/\text{HCO}_3^-$ inside the cell. Airway epithelial cells are exposed to changing concentrations of CO_2 and HCO_3^- . The airway acidifies in airway disease exacerbations as shown by low pH in exhaled breath condensate in exacerbations of asthma (Hunt et al., 2000; Ojoo et al., 2005), COPD, and bronchiectasis (Kostikas et al., 2002). Inflammation causes persistent intra- and extracellular airway acidification similar to arthritic joints (Andersson et al., 1999), possibly through cytokine-mediated, increased activity of a dual NADPH oxidase expressed in airway epithelial cells that produces intracellular H^+ (Schwarzer et al., 2004). Since acidification itself has a negative effect on CBF (Sutto et al., 2004), compensation mechanisms involving activation of sAC are possibly critical for adequate function of airway epithelial cells in vivo. In support of this notion, a recent publication has shown interactions of sAC activity and the cystic fibrosis transmembrane conductance regulator (CFTR) in an airway epithelial cell line (Wang et al., 2005) and in the corneal epithelium (Sun et al., 2003).

Since sAC has been shown to regulate sperm motility and since one of the major functions of airway epithelial cells is to maintain adequate mucociliary clearance through ciliary activity, we wanted to explore whether sAC is expressed in airway epithelial cells and whether it

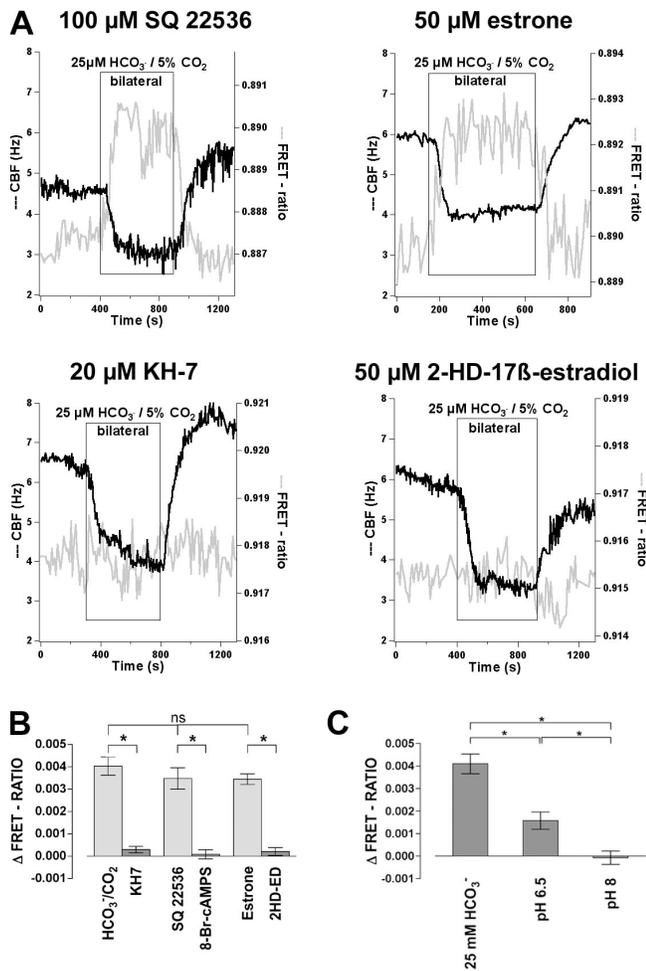


Figure 7. Real-time measurements of simultaneous changes of fluorescently labeled PKA FRET ratio and CBF in human airway epithelial cells. (A) Upon bilateral perfusion of basolaterally permeabilized ALI cultures with 25 mM $\text{HCO}_3^-/5\% \text{CO}_2$, decreases of CBF occur simultaneously with cAMP production indicated by an increase in FRET ratio (PKA dissociation) in the presence of SQ22536 (a tmAC inhibitor with no influence on sAC) and estrone (an estrogen compound with no effect on sAC). In contrast, CBF decreases significantly more and no cAMP is produced upon bilateral perfusion of basolaterally permeabilized ALI cultures with 25 mM $\text{HCO}_3^-/5\% \text{CO}_2$ in the presence of KH7 (a specific sAC inhibitor) and 2-hydroxy-17 β -estradiol (a sAC inhibitor). Thus, cAMP production due to sAC stimulation partially rescues the pH-mediated decrease of CBF upon $\text{HCO}_3^-/\text{CO}_2$ exposure. (B) Statistical analysis shows that the increase in FRET ratio was blocked by 2-hydroxy-17 β -estradiol (2HD-ED) and KH7 (both sAC inhibitors) as well as 8-Br-cAMPS (PKA dissociation inhibitor), but not with SQ22536 (tmAC inhibitor) and estrone (inactive estradiol analogue). (C) To exclude that pH alone influenced FRET ratio (by decreasing YFP fluorescence during acidification), experiments were repeated with basolaterally permeabilized ALI cultures and bilateral perfusion with solutions of pH 6.5 or 8. The pH of 6.5 was chosen since acidification of the cells upon $\text{HCO}_3^-/\text{CO}_2$ exposure results in a pH_i of ≥ 6.5 as estimated with BCECF (Sutto et al., 2004). Using pH 6.5 resulted in a significantly lower increase in FRET ratio compared with 25 mM HCO_3^- while pH 8.0 had no effect. *, $P < 0.05$, comparing groups indicated by brackets; ns, not significant. All $n = 12$ in B and C. The maximal increases in FRET ratio shown in B (~ 0.004) correspond to increases in [cAMP] of $\sim 50 \mu\text{M}$ (Schmid et al., 2006).

is involved in regulating ciliary beating upon changes in $[\text{CO}_2/\text{HCO}_3^-]$. We have previously shown that changes in intracellular pH (pH_i) in the absence of HCO_3^- directly modify CBF (Sutto et al., 2004) and that intracellular acidification decreases CBF. Since biological systems contain $\text{CO}_2/\text{HCO}_3^-$ buffers, it is likely that the cell will try to counterbalance a decrease in pH_i through influx or production of HCO_3^- . Under these conditions, sAC would be activated and produce cAMP, a second messenger known to stimulate CBF via activation of an axonemal PKA (e.g., Sanderson and Dirksen, 1989; Lansley et al., 1992; Kultgen et al., 2002; Wyatt et al., 2005; Schmid et al., 2006). Thus, this signaling is expected to counterbalance the negative effects of acidification on ciliary beating.

Our data presented here show that sAC is in fact expressed in airway epithelial cells. RT-PCR revealed three variants, two of which have been previously reported. It remains unclear whether any of these variant transcripts give rise to polypeptides with distinct enzymatic activity (Geng et al., 2005) but two of them miss part of the first catalytic subunit. Western blotting using polyclonal antibodies revealed that three known variants of sAC (Jaiswal and Conti, 2001; Geng et al., 2005) were present in airway epithelial cells, resulting in immunopositive bands at $\sim 50, 75$, and 190 kD as described before in other cells (Chen et al., 2000; Geng et al., 2005). Most interestingly, however, cilia contained only the 50-kD form, which was depleted in deciliated cells (Fig. 2). It remains unclear why and how this isoform is so specifically localized to the axoneme while the other forms remain in the cell body.

The results obtained by Western blotting with the polyclonal antibodies were confirmed using a monoclonal antibody (R41) (Zippin et al., 2003). Immunofluorescence showed specific staining associated with cilia and the apical pole of airway cells both in sections from human tracheas as well as in airway epithelial cells cultured and fully redifferentiated at the ALI.

While the expression and localization data were convincing, it was much harder to show the relevance of sAC activity to ciliary beating. The reason for this problem was the sensitivity of CBF to changes in pH_i , independent of $\text{CO}_2/\text{HCO}_3^-$ (Sutto et al., 2004). We therefore had to dissect CBF responses to pH (which are independent of cAMP and PKA) from responses to $\text{CO}_2/\text{HCO}_3^-$ (which are presumably dependent on sAC, cAMP, and PKA). Since it is not possible to measure $[\text{CO}_2]$ or $[\text{HCO}_3^-]_i$ changes in real time, experiments with inhibitors of sAC and PKA were used instead.

To achieve changes in $[\text{CO}_2]$ and $[\text{HCO}_3^-]_i$, we executed a series of apical and basolateral perfusion changes using airway epithelial cells cultured and redifferentiated at the ALI. First, the apical perfusate was changed in nonpermeabilized cells to a solution buffered with 25 mM $\text{HCO}_3^-/5\% \text{CO}_2$, while the basolateral perfusion was maintained with a HEPES-buffered, no HCO_3^- -containing

solution. Here, the suspected sAC activation by $\text{HCO}_3^-/\text{CO}_2$ was revealed by showing that inclusion of the specific sAC inhibitor KH7 (Fig. 4) resulted in a larger degree of CBF decrease.

We repeated these experiments with basolaterally permeabilized airway epithelial cells grown and differentiated at the ALI. First, the apical perfusate was switched from a HEPES-buffered solution to a 25 mM $\text{HCO}_3^-/5\%\text{CO}_2$ -buffered solution. Again, there was a decrease in CBF likely due to a decrease in pH_i from CO_2 influx that could not be buffered by the basolateral, HEPES-containing solution. The decrease in CBF, however, was larger upon inhibition of PKA by H89 and of sAC by the specific inhibitor KH7. When the basolateral solution was subsequently exchanged with one containing 25 mM $\text{HCO}_3^-/5\%\text{CO}_2$, CBF increased and this increase was again inhibited by H89 and KH7. Thus, these results strongly suggested that CBF is sensitive to changes in $\text{CO}_2/\text{HCO}_3^-$ that stimulate sAC to produce cAMP, which in turn activates PKA.

To finally show that cAMP is indeed produced by these cells in response to changing HCO_3^- , we used ciliated airway epithelial cells expressing fluorescently tagged PKA subunits that were introduced into undifferentiated cells via infection with recombinant, replication-deficient HIV-pseudotyped lentiviruses driving the expression of the fusion proteins with the ciliated cell-specific promoter *foxj1* (Schmid et al., 2006). These cells allow direct measurement of PKA activation by changes in the FRET ratio between the regulatory subunit (RII) labeled with CFP and the catalytic subunit labeled with YFP. This activation or change in FRET is directly proportional to [cAMP] (Zaccolo and Pozzan, 2002; Schmid et al., 2006). These experiments not only confirmed cAMP production in ciliated cells upon increases in $\text{CO}_2/\text{HCO}_3^-$, they also showed that $\text{CO}_2/\text{HCO}_3^-$ -mediated cAMP production was inhibited with sAC inhibitors, including KH7 and 2-hydroxy-17 β -estradiol, but not with the tmAC inhibitor SQ22536 or the inactive 2-hydroxy-17 β -estradiol analogue estrone. SQ22536 on the other hand both blocked cAMP production and CBF changes upon stimulation of the cells with forskolin, a tmAC inhibitor, while KH7 had no effect on these responses. Further specificity of the FRET ratio changes was confirmed by using a competitive inhibitor of PKA (blocking its dissociation), 8-Br-CAMPS, which inhibited tagged PKA activation (Fig. 7 B).

Since decreases in pH reduce the fluorescence of YFP, thereby possibly mimicking an increase in the FRET ratio, we also examined the direct effect of pH on FRET in basolaterally permeabilized cells (in the absence of HCO_3^-). Since 25 mM $\text{HCO}_3^-/5\%\text{CO}_2$ results in an approximate intracellular pH decrease of 0.5 units (Sutto et al., 2004), pH_i was changed to 6.5 using basolateral perfusion. While this pH change slightly increased apparent FRET ratio, the increase was significantly lower than the increase seen upon $\text{CO}_2/\text{HCO}_3^-$ perfusion.

Since the inhibitor experiments also blocked the change in FRET ratio, these data strongly suggest that the recorded FRET ratio change is a true reflection of changes in intracellular cAMP and not of changes in pH.

In summary, we have shown that sAC is expressed in airway epithelial cells and that at least one particular isoform is localized to the axoneme. In addition, we show functional evidence of involvement of sAC in ciliary beat regulation. Finally, we show real-time evidence for reversible, HCO_3^- -mediated cAMP production in ciliated cells using cAMP-reporter constructs in living cells on a real-time basis, confirming the importance of sAC-mediated cAMP production in intact cells.

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