# Real-time analysis of cAMP-mediated regulation of ciliary motility in single primary human airway epithelial cells

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## Summary

**Airway ciliary beat frequency regulation is complex but in part influenced by cyclic adenosine monophosphate (cAMP)-mediated changes in cAMP-dependent kinase activity, yet the cAMP concentration required for increases in ciliary beat frequency and the temporal relationship between ciliary beat frequency and cAMP changes are unknown. A lentiviral gene transfer system was developed to express a fluorescence resonance energy transfer (FRET)-based cAMP sensor in ciliated cells. Expression of fluorescently tagged cAMP-dependent kinase subunits from the ciliated-cell-specific *foxj1* promoter enhanced expression in fully differentiated ciliated human airway epithelial cells, and permitted simultaneous measurements of ciliary beat frequency and cAMP (represented by the FRET ratio). Apical application of forskolin (1 μM, 10 μM, 20 μM) and, in permeabilized cells, basolateral cAMP (20 μM, 50 μM, 100 μM) caused dose-dependent, albeit similar and simultaneous – increases in cAMP and ciliary beat frequency. However, decreases in cAMP preceded decreases in ciliary beat frequency, suggesting that either cellular cAMP decreases before ciliary cAMP or the dephosphorylation of target proteins by phosphatases occur at a rate slower than the rate of cAMP hydrolysis.**

Key words: cAMP, Ciliary beating, Protein kinase A, Kinetics relationship, Air-liquid interface

## Introduction

There is ample evidence that cyclic adenosine monophosphate (cAMP) regulates mammalian ciliary beat frequency (CBF). Agonists of $G\alpha_s$-coupled receptors and forskolin that increase intracellular cAMP through stimulation of transmembrane adenylyl cyclases (tmACs) all stimulate CBF, and these effects can be blocked with inhibitors of cAMP-dependent protein kinase A (PKA) (Di Benedetto et al., 1991; Frohock et al., 2002; Lansley et al., 1992; Sanderson and Dirksen, 1989; Tamaoki et al., 1989b; Verdugo et al., 1980; Wyatt et al., 1998; Yang et al., 1996). In analogy to the situation in *Paramecium* (Hamasaki et al., 1991; Hamasaki et al., 1989), cAMP possibly regulates CBF by activating PKA localized to the axoneme by its A kinase anchoring protein (Kultgen et al., 2002). PKA in turn phosphorylates a dynein light chain (Kultgen et al., 2002; Salathe et al., 1993; Sisson et al., 2000). Even though cells apically permeabilized using saponin have not been found to change ciliary beating upon addition of cAMP (Lansley et al., 1992), isolated axonemes have been shown to beat faster when cAMP levels are increased (Wyatt et al., 2005). The differences in these findings remain unclear, but suggest some role for the ciliary membrane and the intracellular environment in the regulation of CBF in addition to cAMP. This view is supported by the finding that patch-clamped ciliated airway epithelial cells with intact ciliary membranes respond with an increase in CBF to a controlled increase of cytosolic cAMP (Ma et al., 2002).

Thus, there is evidence for a role of cAMP in mammalian CBF regulation but the temporal correlation between changes in CBF and cAMP has not been explored. cAMP measurements in single live cells have been accomplished with fluorescence resonance energy transfer (FRET)-based methods. Fluorescently labeled subunits of PKA, that dissociate upon cAMP binding and reduce FRET, have been injected into single cells (Adams et al., 1991) or expressed as fusion proteins after transfections of plasmids into primary cells (Zaccolo et al., 2000; Zaccolo and Pozzan, 2002). An additional FRET-based cAMP reporter has been described by two groups using the cAMP-binding protein Epac or guanine nucleotide exchange factor for Rap1 (DiPilato et al., 2004; Nikolaev et al., 2004). Finally, an alternative cAMP measurement was reported by expressing recombinant cAMP-gated channels (Rich et al., 2001). None of these methods is easily accomplished with primary airway ciliary epithelial cells, submerged or re-differentiated at the air-liquid interface (ALI) because of the difficulties of injecting, transfecting or infecting differentiated airway epithelial cells.

Here, we show the simultaneous, real-time correlations of changes in CBF and estimated intracellular cAMP concentration $[cAMP]_i$ in single, ciliated human airway

epithelial cells using FRET, and a lentivirus-based infection strategy of undifferentiated airway epithelial cells and targeted expression of the fluorescently tagged PKA subunit proteins to ciliated cells after differentiation. Our data reveal that activation of PKA coincides with the increase in CBF, whereas the return of CBF to baseline lags behind PKA deactivation, indicating an important role for phosphatases to stop the PKA-mediated stimulation of ciliary beating.

## Results

### NCI-H292 cells

To explore the temporal relationship between changes in cAMP and CBF, FRET-based reporters that use fluorescently tagged PKA subunits were cloned into recombinant lentiviruses. Expression of the reporter constructs was driven by the cytomegalovirus (CMV) promoter. One lentivirus encoded the PKA catalytic subunit (CAT) fused with yellow fluorescent protein (YFP) (CAT-YFP) and the other virus encoded the PKA regulatory subunit type 2 (RII) fused with cyan fluorescent protein (CFP) (RII-CFP). When $[cAMP]_i$ is low, holotetrameric PKA provides maximal FRET between the fluorescently tagged PKA subunits. When $[cAMP]_i$ rises, separation of RII and CAT reduces FRET, and thus increases the fluorescence ratio of the emitted light from CFP over YFP, a change proportional to changes in $[cAMP]_i$ (Zaccolo et al., 2000; Zaccolo and Pozzan, 2002).

NCI H292 cells were infected with both lentiviruses at an multiplicity of infection (MOI) of 0.8 for each virus. Fluorescence could be detected between 24 and 72 hours after infection. The infection rate for each of the viruses, as assessed by detection of YFP or CFP fluorescence (Fig. 1), was ~30%. The rate of dual infection was only 5-10%.

The co-infected NCI H292 cells were used to measure FRET ratios as an estimate of changing $[cAMP]_i$. Forskolin, which activates tmACs, was applied to increase $[cAMP]_i$. As shown in Fig. 1, the increased FRET ratio in single cells upon stimulation by forskolin, was reversible when forskolin was removed, and could be repetitively increased upon additional addition and removal of forskolin. No calibration with cAMP was attempted in these cells.

### Expression of tagged PKA subunits in primary airway epithelial cells with the CMV promoter

The infection of undifferentiated primary human airway epithelial cells with lentiviruses that drive the expression of RII-CFP and CAT-YFP subunits through the CMV promoter was about as efficient in as in NCI H-292 cells, showing that this lentiviral system efficiently infects these cells. In addition, the levels of expression were similar in the two cell types; however, the number of primary airway epithelial cells expressing the constructs decreased after day 5 as the cells differentiated, when they were apically exposed to air by establishing the ALI (Fig. 2). In fully differentiated cultures, 10-20% of the cells expressed either CFP- or YFP-tagged fusion proteins showing ~50% co-expression. Ciliated and non-ciliated cells were both able to express either protein (expression efficiency ~50% for each protein). Thus, many ciliated cells did not express any tagged PKA subunits and only a very limited number of ciliated cells were identified expressing both fusion proteins. Simultaneous recordings of CBF and FRET are difficult but FRET signals were measured from ciliated cells expressing both tagged PKA subunits. Again, FRET ratios increased and decreased repetitively in single cells upon addition and removal of forskolin, indicating that the lentiviruses properly expressed the PKA subunits, the subunits were assembled in primary human airway epithelial

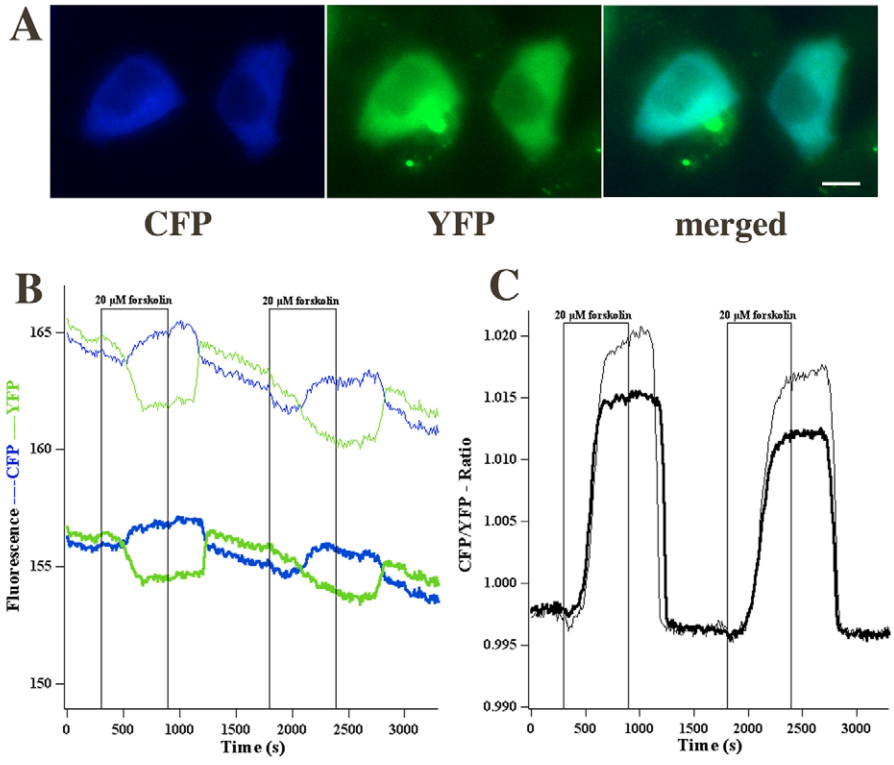


**Fig. 1.** Estimation of $[cAMP]_i$ changes using FRET in NCI-H292 cells. NCI-H292 cells were co-infected with HIV-derived lentiviruses encoding the fluorescently labeled subunits of PKA driven by the CMV promoter. (A) FRET was demonstrated by exciting cells at 436 nm and visualizing CFP emission at 480 nm (left panel, blue) and YFP emission at 535 nm from a pair of cells (middle panel, green). Right panel shows merged image. Bar, 10 μm. (B) Repeated stimulation of the two cells pictured in A (left cell, thin lines; right cell, thick lines) with 20 μM forskolin increased CFP (blue trace) and decreased YFP emission (green), consistent with a reduction in FRET due to separation of the PKA subunits. (C) Ratio data (CFP:YFP fluorescence; left cell: thin line and right cell, thick line) reveal reversible increases in estimated $[cAMP]_i$ upon exposure to 20 μM forskolin.

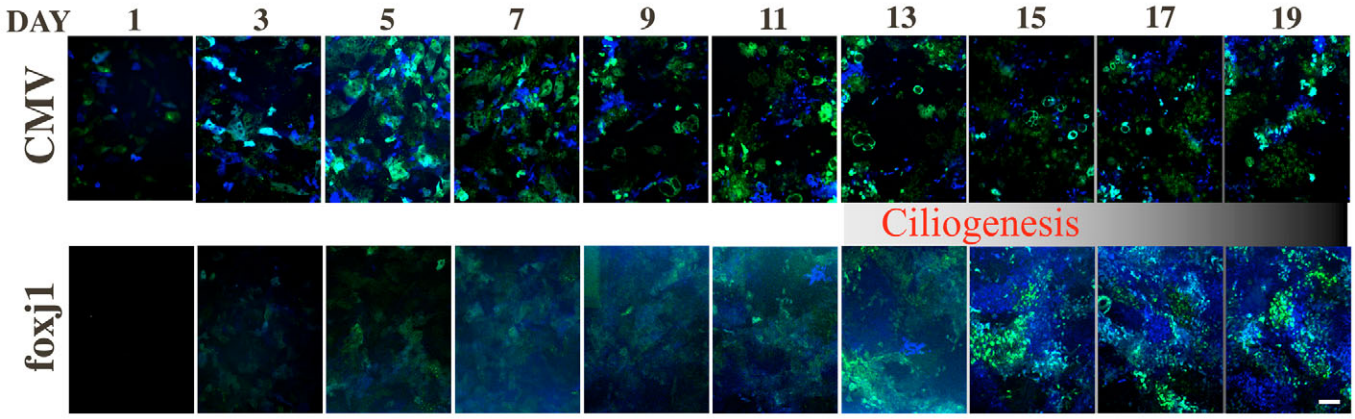


**Fig. 2.** Time course of PKA subunit expression using CMV or *foxj1* promoter in human airway epithelial cells. Undifferentiated cells were infected and then re-differentiated on T-clear filters at the ALI and imaged every other day for 19 days. Pictures are merged images obtained by first exciting CFP at 436 nm, recording the emitted light at 480 nm, and then exciting YFP at 510 nm, recording the emitted light at 535 nm. (Upper row) CMV promoter. Using this promoter, rapid expression of both fluorescently tagged PKA subunits was seen, reaching a maximal expression around day 5. (Lower row) *foxj1* promoter. Using the this promoter, expression of both fluorescently tagged PKA subunits was initially low but increased markedly around day 11 together with the beginning of ciliogenesis as assessed by phase-contrast microscopy (semi-quantitatively shown by the gradient between the rows getting darker from white to black). All images were taken with the same camera settings and a standard excitation time. Bar, 100 μm. Cultures were from a single lung donor and matched with regard to culture and infection conditions.

cells and dissociated upon stimulation of cAMP synthesis. No calibration with cAMP was attempted in these cells either because of the low number of ciliated cells expressing both proteins.

### Ciliated-cell-specific expression of tagged PKA subunits using the *foxj1* promoter

To increase the overall frequency of expression (and thereby the frequency of dually expressing ciliated cells), the CMV promoter in the lentivirus constructs was exchanged for the ciliated-cell-specific *foxj1* promoter, which has previously been proven to be effective in murine and human ciliated airway epithelial cells (Ostrowski et al., 2003). After infection with these new lentiviral constructs, faint CFP and YFP fluorescence was seen in some undifferentiated cells after 24-48 hours. After 10-12 days of differentiation on air and with the beginning of ciliation, however, the fluorescence intensity of both PKA-subunit fusion proteins increased markedly as the cells differentiated to a maximum at the time of full ciliation, as assessed by phase-contrast microscopy (Fig. 2). This was in contrast to the CMV-driven constructs, where the early onset of fluorescence, beginning the day after infection, was peaking at day 5, after which the expression declined (Fig. 2). Using cells that express constructs through the *foxj1* promoter, 80-90% of ciliated cells were positive for either the CFP- or YFP-tagged fusion protein, with almost 100% co-expression.

Staining cilia in cultures with anti-acetylated tubulin antibodies revealed expression through the CMV promoter in both ciliated and non ciliated cells, whereas the *foxj1* promoter is exclusively active in ciliated cells (Fig. 3). *Z*-stack images of ALI cultures obtained by confocal microscopy revealed that fluorescently tagged PKA subunits expressed by using the *foxj1* promoter were localized in ciliated cells. Both subunits were localized mainly to the apical compartment, including cilia (Fig. 4), identical to the location of endogenously expressed CAT and RII subunits of PKA (Kultgen et al., 2002).

### Measurement of changes in estimated $[cAMP]_i$ and CBF in single ciliated cells

Airway epithelial cells that had been infected with *foxj1*-promoter-driven lentiviral constructs in an undifferentiated state and then fully differentiated at the ALI, were used to simultaneously measure single-cell changes in estimated $[cAMP]_i$ by FRET and also in CBF by infrared differential interference contrast (IR-DIC) video microscopy. Intact ciliated cells were apically exposed to increasing concentrations of forskolin. In addition, basolaterally permeabilized cells were used for simultaneous measurements of CBF and estimated $[cAMP]_i$, while being perfused basolaterally with cAMP solutions of different concentration to calibrate FRET ratios in relation to known $[cAMP]_i$ and apically with forskolin. It needs to be emphasized that the basolateral solution used for these experiments with permeabilized cells contained ATP so that cilia were able to beat. Figs 5 and 6 show examples of such measurements, whereas Fig. 7 summarizes the data of all experiments.

### CBF and FRET responses to forskolin exposure of non-permeabilized ciliated cells

The apical side of ciliated cells, which expressed the recombinant, tagged PKA subunits, was transiently exposed to increasing concentrations of forskolin (exposure time was always 300-400 seconds with subsequent washout). CBF baselines before each forskolin addition were statistically not significantly different: 6.1±0.2 Hz ($n$=13) before 1 μM forskolin, 6.5±0.1 Hz before 10 μM forskolin ($n$=23) and 6.5±0.2 Hz ($n$=28) before 20 μM forskolin (all $P$>0.05). Forskolin exposure increased CBF transiently to a maximum of 19.1±1.7%, 26.1±1.5% and 30.5±1.9% above baseline upon 1 μM, 10 μM and 20 μM forskolin, respectively. All increases were statistically significant ($P$<0.05). The rates of the CBF increase grew with each forskolin increment (0.32±0.03 Hz/minute, 0.8±0.07 Hz/minute and 1.25±0.13 Hz/minute for

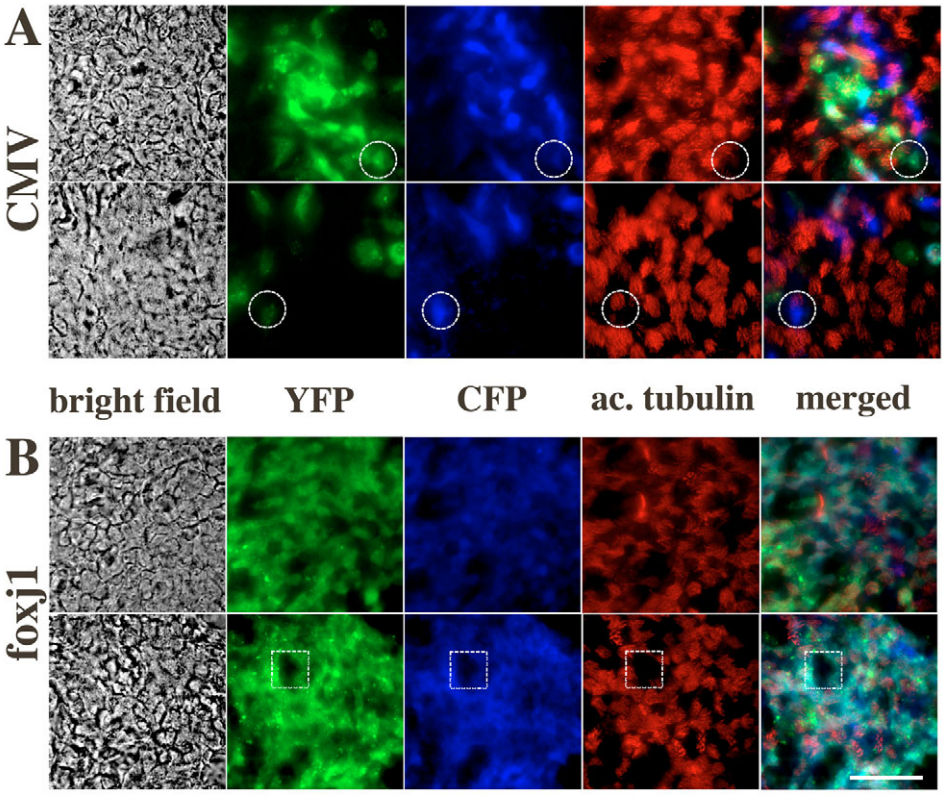


**Fig. 3.** Ciliated cell expression of PKA fusion constructs in infected and fully differentiated human airway epithelial cells. (A,B) Undifferentiated cells were infected with lentiviruses expressing the PKA subunit fusion proteins from either the CMV promoter or the *foxj1* promoter. Dually infected and differentiated cells were stained with anti-acetylated α tubulin (ac. tubulin) for cilia with an Alexa Fluor-555-coupled secondary antibody. In cells expressing constructs with (A) the CMV promoter, 10-20% of the cells express either the CFP- or YFP-tagged fusion proteins, of which 50% of cells coexpression both fusion proteins. Both ciliated and non-ciliated cells express the proteins (~50% each). Circles indicate fields with little or no cilia signal but with expression of tagged fusion proteins. In cells expressing constructs with (B) the *foxj1* promoter, 80-90% of ciliated cells express either the CFP- or YFP-tagged fusion proteins with promoter with almost 100% coexpression. No expression is seen in non-ciliated cells (squares). Bar, 50 μM. Cultures were from a single donor and matched with regard to all culture and infection conditions.

1 μM, 10 μM and 20 μM forskolin, respectively; all $P<0.05$ compared with each other), indicating that higher forskolin concentrations increase CBF more rapidly compared with lower concentrations.

From the same cells, changes in $[cAMP]_i$ were estimated by measuring FRET ratios. Forskolin exposure increased the FRET ratio transiently by 0.0017±0.0002 arbitrary units (AU), 0.0029±0.0004 AU and 0.0041±0.0003 AU upon 1 μM, 10 μM

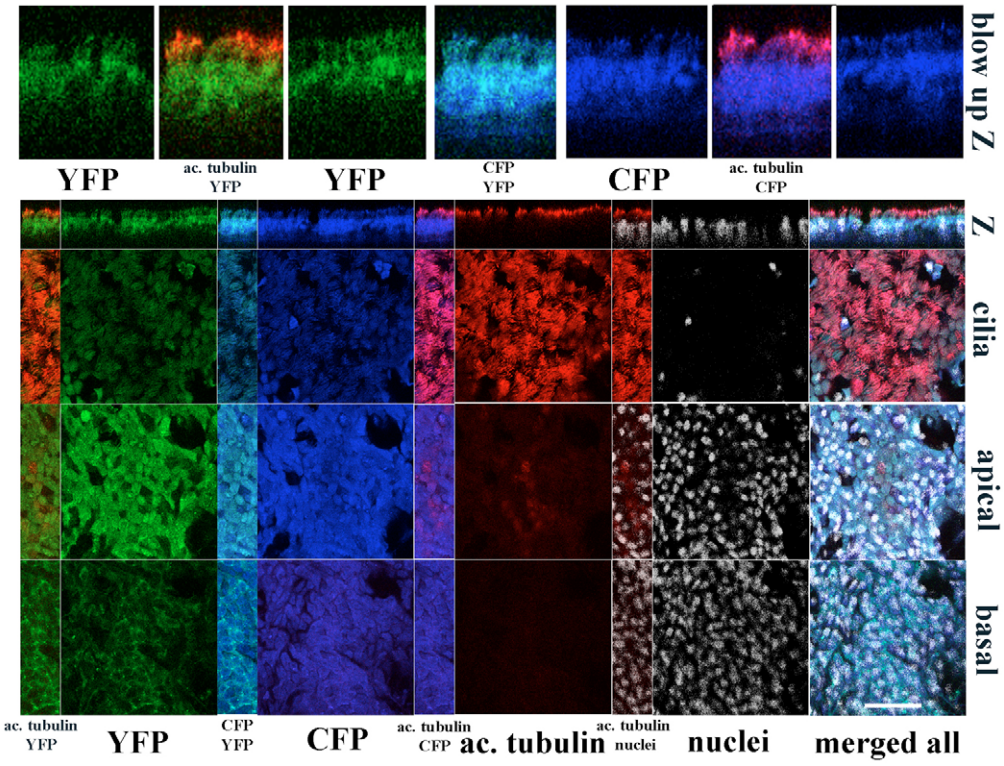


**Fig. 4.** Apical localization of PKA fusion constructs in infected and fully differentiated human airway epithelial cells. Fully differentiated cells were infected in their undifferentiated state with *foxj1* promoter-containing lentiviral constructs and stained for cilia with anti-acetylated α tubulin (ac. tubulin) and an Alexa Fluor-555-coupled secondary antibody (red) and with DAPI for nuclei (pseudo-colored in white). (A) Magnified *z*-axis reconstructions showing expression of both fusion proteins in cilia. (B) *Z*-axis reconstructions and *xy* cuts through different levels of the cultures corresponding to cilia, apical and basal cell compartments are shown. Channels are labeled at the bottom including overlay images. Both CFP- and YFP-labeled PKA subunits were mainly localized to the apical compartment of the cells and they can be easily seen inside cilia. Bar, 50 μm.

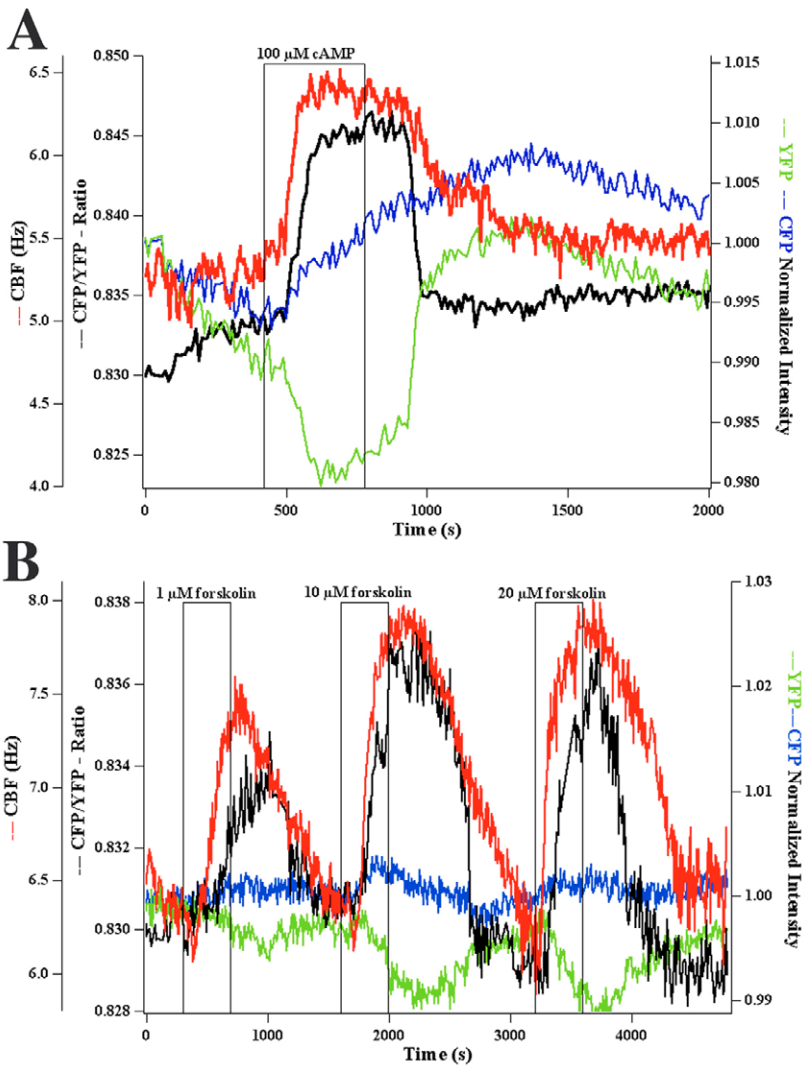


**Fig. 5.** Real-time measurements of simultaneous changes of YFP- and CFP-intensities, FRET-ratio and CBF in single human airway epithelial cells. (A) Dually infected and differentiated cells were basolaterally permeabilized and basolaterally perfused. Intensities of CFP (blue) and YFP (green) emissions were measured during CFP excitation simultaneously with CBF (red). FRET ratio (black) was calculated as quotient of the intensities CFP/YFP. Addition of cAMP to the perfusate is indicated with a bar above the traces. (B) Dually infected and differentiated cells were imaged without permeabilization. Intensities of CFP (blue) and YFP (green) emissions were measured during CFP excitation simultaneously with CBF (red). The FRET ratio (black) was calculated during perfusion of the apical surface with sequential solutions containing 1 μM, 10 μM and 20 μM forskolin.

and 20 μM forskolin exposures, respectively (all $P<0.05$ compared with each other). The rises in FRET ratio and CBF occurred within the time resolution of the FRET measurement (10 seconds). Increasing the time resolution led to unacceptable bleaching of the fluorophores and was therefore not feasible. The rates of the FRET ratio increase in response to forskolin was 0.0004±0.00004 per minute, 0.0008±0.00008 per minute and 0.0011±0.001 per minute for 1 μM, 10 μM and 20 μM forskolin, respectively (all $P<0.05$ compared with each other), indicating that higher forskolin concentrations, as with the CBF measurements, increase the FRET ratio more rapidly compared to lower concentrations.

In summary, increases in FRET ratio and CBF upon forskolin exposure occurred at the same time (within the time resolution of the FRET ratio measurements), and the rate of change of both signals increased with increasing forskolin concentrations.

To make sure that the recombinant PKA expression did not influence the results, we used fully differentiated ciliated cells (lung donor cells matched to those used for FRET) that did not express recombinant PKA and exposed those cells apically to forskolin. CBF baselines before each forskolin addition were statistically not different from each other and, as a whole, not different from cells expressing recombinant PKA (6.7±0.2 Hz, $n=10$). Forskolin exposure increased CBF transiently by 17.1±3.8%, 25.8±3.9% and 30.9±4.6% above baseline upon exposure with 1 μM, 10 μM and 20 μM forskolin, respectively. Although all increases were statistically significant (all $P<0.05$ compared with baseline and each other), they were not different from cells expressing recombinant PKA (all $P>0.05$). The rate of CBF increase in response to forskolin was

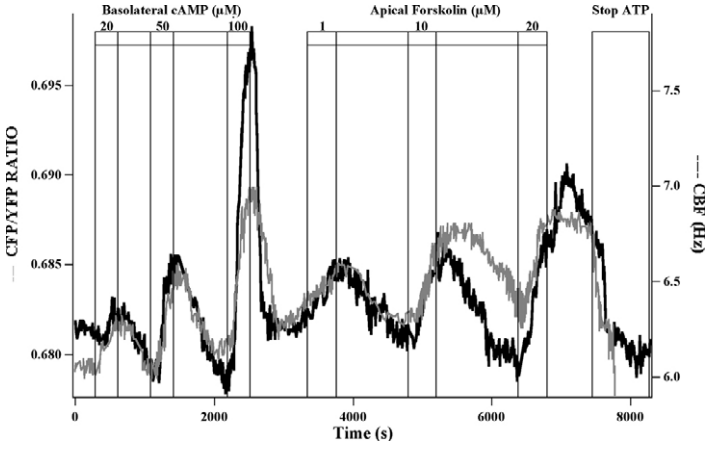


**Fig. 6.** Dose response of FRET ratio and CBF to cAMP and forskolin in permeabilized human airway epithelial cells. FRET ratio (black) and CBF changes (grey) were recorded from a single ciliated cell, in a previously permeabilized culture that was sequentially perfused on the basolateral side with 20 μM, 50 μM and 100 μM cAMP. Subsequent apical perfusion with 1 μM, 10 μM and 20 μM forskolin are shown. At about 7500 seconds ATP was removed from the basal chamber, which caused a rapid fall of CBF, confirming successful permeabilization of the cell. Thus, this last example does not show the delay of the CBF return to baseline compared to baseline with 20 μM forskolin (see Fig. 9).

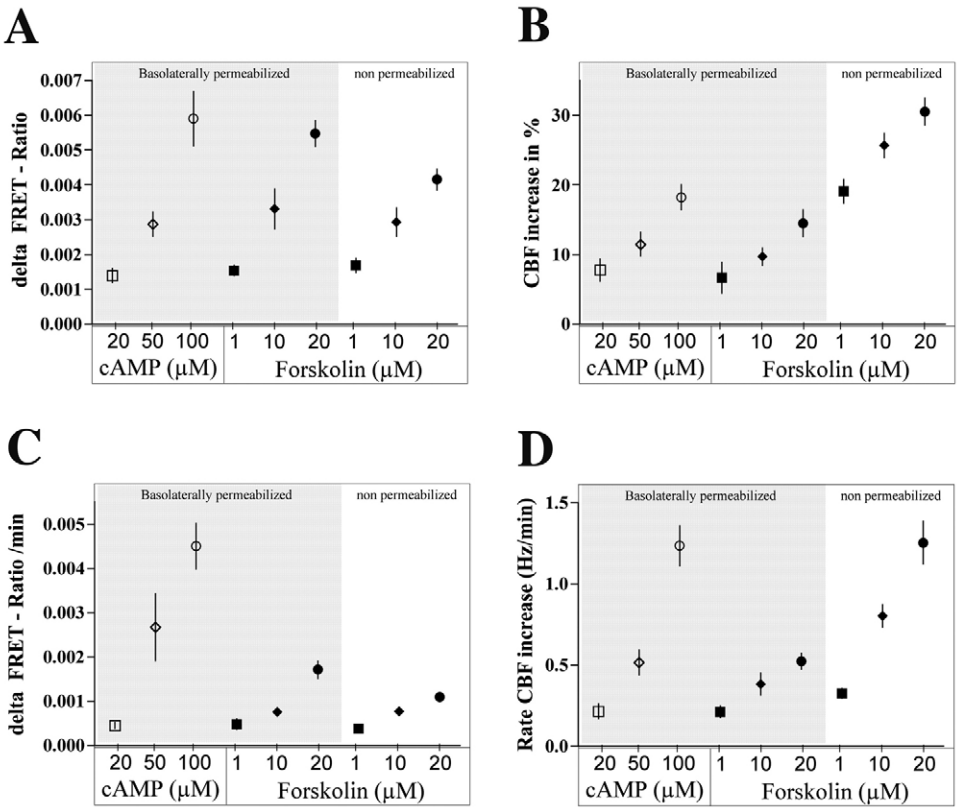


**Fig. 7.** Calibration of cAMP and forskolin induced FRET ratio and CBF. Basolaterally permeabilized cells or non-permeabilized cells were perfused apically with forskolin, or basolaterally with cAMP. (A) Increases in FRET ratio (in AUs), (B) increases in CBF (% above baseline), (C) rate of the FRET ratio increases and (D) rate of CBF changes are shown for all stimulations. All results stem from at least eight cells from two different lungs in two different experiments (see text for more details). Values are plotted as the mean ± s.e.m. □, 20 μM cAMP; ◇, 50 μM cAMP; ○, 100 μM cAMP; ■, 1 μM forskolin; ◆, 10 μM forskolin; ●, 20 μM forskolin. See text for significance levels.

0.26±0.04 Hz/minute, 0.84±0.14 Hz/minute and 1.21±0.12 Hz/minute for 1 μM, 10 μM and 20 μM forskolin, respectively (all $P<0.05$ compared to each other). These rates did also not differ from those of cells expressing recombinant PKA (all $P>0.05$) and were again consistent with the notion that higher forskolin concentrations increase CBF more rapidly compared with lower concentrations.

### CBF and FRET responses of basolaterally permeabilized ciliated cells exposed apically to forskolin

Cells were basolaterally permeabilized in an effort to calibrate the FRET-ratio signal with known concentrations of cAMP (basolateral perfusates contained ATP to maintain ciliary beating; see Table 1). First, apical application of forskolin (as described above) was examined to assess the effects of permeabilization on CBF and FRET ratios. CBF baselines of permeabilized cells before each forskolin addition were statistically not different from each other ($P>0.05$) but lower than in non-permeabilized cells ($P<0.05$). Baselines were 6.42±0.18 Hz ($n=4$), 5.53±0.34 Hz ($n=11$) and 5.84±0.2 Hz ($n=10$) before 1 μM, 10 μM and 20 μM forskolin, respectively (all $P>0.05$ compared with each other). Forskolin exposure increased CBF transiently by 6.7±2.2%, 9.7±1.3% and 14.5±1.9% above baseline for 1 μM, 10 μM and 20 μM forskolin, respectively. All increases were statistically significant compared with baseline but significantly lower compared with non-permeabilized cells. The rate of CBF increase in response to forskolin was 0.21±0.04 Hz/minute, 0.38±0.07 Hz/minute and 0.52±0.05 Hz/minute for 1 μM, 10 μM and 20 μM forskolin, respectively. As in non-permeabilized cells, these rates increased significantly from 1 μM to 20 μM forskolin ($P<0.05$). In addition, the rates were not significantly different at 1 μM ($P>0.05$), but significantly less at 10 μM and 20 μM compared with those obtained in non-permeabilized cells ($P<0.05$), possibly indicating that the cytosolic environment of these permeabilized cells is somewhat different from non-permeabilized cells.

From the same cells, FRET ratios were measured upon apical forskolin exposure. FRET ratio increased by

**Table 1. Composition of solutions**

| Solution | 1 | 2 | 3 |
|---|---|---|---|
| Application | Apical | Basolateral | Basolateral without ATP |
| NaCl | 117 | 10 | 10 |
| KCl | 5.3 | – | – |
| Na-gluconate | 25 | – | 10 |
| K-gluconate | – | 140 | 140 |
| $CaCl_2$ | 1.3 | 0.45* | 0.1 |
| $MgCl_2$ | 0.5 | – | – |
| $MgSO_4$ | 0.4 | – | – |
| $Na_2HPO_4$ | 0.3 | – | – |
| $KH_2PO_4$ | 0.4 | – | – |
| Glucose | 5.6 | – | – |
| Hepes | 20 | 20 | 20 |
| Mg-ATP | – | 10 | – |
| CrP | – | 10 | – |

Concentrations of solution components are given in mM, pH adjustment was with NaOH (S1, pH 7.4) or KOH (S2,3; pH 7.2). Non-permeabilized cells were perfused apically and basolaterally with S1. Permeabilized cells were perfused apically with S1 and basolaterally with S2,3. *Approximate concentration of free $Ca^{2+}$ after chelation by ATP is 0.1 mM. CrP, creatine phosphate disodium salt.

0.0015±0.0001 AU, 0.0034±0.0006 AU and 0.0055±0.0004 AU upon 1 μM, 10 μM and 20 μM forskolin exposure, respectively (all $P<0.05$ compared with each other), and decreased to baseline after removal of forskolin. The rises in FRET ratio and CBF again occurred within the time resolution of the FRET measurement. The rate of the FRET ratio increase in response to forskolin was 0.0005±0.00001 per minute, 0.0008±0.00005 per minute and 0.0017±0.0002 per minute for 1 μM, 10 μM and 20 μM forskolin, respectively, not significantly different from the rates obtained in non-permeabilized cells.

### CBF and FRET responses to basolateral exposure of cAMP of basolaterally permeabilized ciliated cells

To calibrate the FRET signal with known cAMP concentrations, basolaterally permeabilized ciliated cells that express recombinant, tagged PKA subunits were exposed basolaterally to increasing concentrations of cAMP. CBF baselines before each cAMP addition were statistically not different from each other and not different of those from the permeabilized cells used for forskolin exposures. Baselines were 5.79±0.21 Hz ($n=15$), 5.68±0.23 Hz ($n=15$) and 5.82±0.22 Hz ($n=14$) before 20 μM, 50 μM and 100 μM cAMP, respectively (all $P>0.05$). cAMP exposure increased CBF transiently by 7.00±1.1%, 11.44±1.72% and 18.2±1.8% above baseline for 20 μM, 50 μM and 100 μM cAMP, respectively. All increases were statistically significant compared with baseline and each other, and responses to increasing concentrations of cAMP (20 μM, 50 μM and 100 μM) were not significantly different from permeabilized cells apically exposed to forskolin (1 μM, 10 μM and 20 μM, respectively).

The rate of CBF increase in response to cAMP was 0.21±0.04 Hz/minute, 0.52±0.08 Hz/minute, and 1.23±0.12 Hz/minute for 20 μM, 50 μM and 100 μM cAMP, respectively (all $P<0.05$). These rates were significantly higher than those obtained in permeabilized cells apically exposed to 1 μM, 10 μM and 20 μM forskolin ($P<0.05$), but not different from non-permeabilized apically exposed to 1 μM, 10 μM and 20 μM forskolin ($P>0.05$). These data indicate that basolateral permeabilization affects signaling at the apical membrane.

From the same permeabilized cells, FRET ratios were measured upon cAMP exposure. FRET ratios increased by 0.0014±0.0002 AU, 0.0028±0.0003 AU and 0.0059±0.0008 AU upon exposure of 20 μM, 50 μM and 100 μM cAMP, respectively (all $P<0.05$ compared with each other), and returned to baseline after cAMP was removed. The rises in FRET ratio and CBF occurred again within the time resolution of the FRET measurement. The rate of the FRET ratio increase in response to cAMP was 0.0005±0.00007 per minute, 0.0027±0.0008 per minute and 0.0045±0.0005 per minute for 20 μM, 50 μM and 100 μM cAMP, respectively (all $P<0.05$). The rates of FRET-ratio changes induced by 50 μM and 100 μM cAMP were significantly higher than those induced after the apical perfusion with 10 μM and 20 μM forskolin of permeabilized and non-permeabilized cells ($P<0.05$). These data indicate that the rise in cAMP due to forskolin (and thereby activation of PKA) is slow and takes considerable time given the FRET-measurement time resolution of 10 seconds.

Overall, 100 μM cAMP caused a statistically identical change in CBF and the FRET ratio compared with 20 μM forskolin. Likewise, 50 μM cAMP yielded identical results to 10 μM forskolin and 20 μM cAMP yielded identical results to 1 μM forskolin. In addition, a clear dose-response relationship was seen both with cAMP and forskolin. Control cells that do not express recombinant PKA subunits, were basolaterally permeabilized and used for basolateral cAMP exposure ($n=8$). The results were statistically indistinguishable from cells that expressed recombinant, tagged PKA subunits.

### Time courses of FRET and CBF changes

To compare the kinetics of the FRET ratio and CBF changes, the time from FRET ratio baseline and CBF baseline to the maximal response was measured in non-permeabilized cells apically perfused with 1 μM, 10 μM and 20 μM forskolin or in permeabilized cells basolaterally perfused with 20 μM, 50 μM and 100 μM cAMP (Fig. 8). In addition, the time from maximal response back to baseline after removal of the stimulus was measured (Fig. 8).

For 1 μM, 10 μM and 20 μM forskolin, the times for increases of CBF vs FRET ratios were 324.7±21.4 seconds vs 322.2±21.4 seconds, 309.6±20.1 seconds vs 290.0±21.0 seconds and 306.3±27 seconds vs 327.8±31.7 seconds, respectively ($n=9$), none of which are significantly different from each other ($P>0.05$). The times for the decrease of CBF vs FRET ratio were 752.0±39.1 seconds vs 533.3±36.2

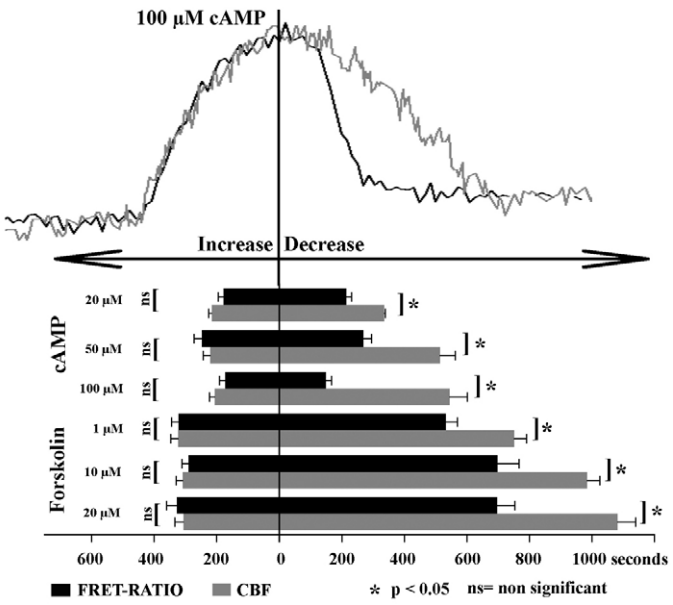


**Fig. 8.** Comparison of the kinetics of FRET ratio and CBF changes during and after stimulation with cAMP or forskolin. Infected and differentiated human airway epithelial cells were either perfused apically with 1 μM, 10 μM or 20 μM forskolin (non-permeabilized cells; $n=9$) or basolaterally with 20 μM, 50 μM or 100 μM cAMP (permeabilized cells; $n=7$). The top of the graph shows an example of a permeabilized cell perfused basolaterally with 100 μM cAMP. The left side of the graph depicts the time to increase FRET-ratio (black) or CBF (gray) from baseline to their maxima in seconds, which were statistically indistinguishable ($P>0.05$). The right side depicts the times for the FRET ratio and CBF to return to baseline from their maxima. In each case, CBF decreased significantly slower than the FRET ratio ($P<0.05$ for all pairs).

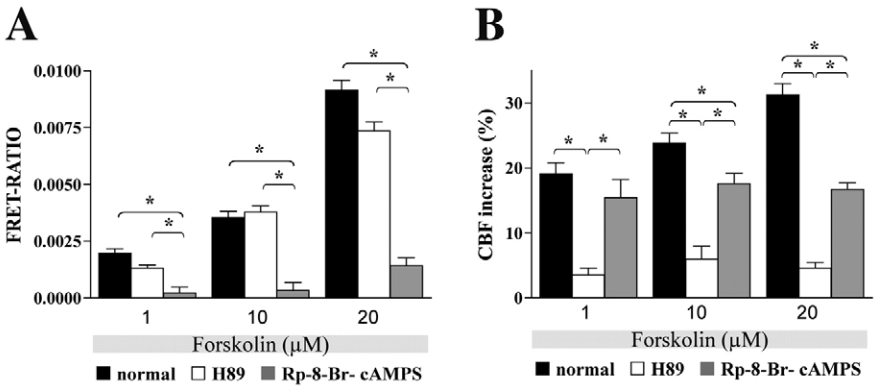


**Fig. 9.** Inhibition of FRET ratio and CBF increases with H89 and Rp-8-Br-cAMPS. Non-permeabilized cells were perfused basolaterally with 400 μM Rp-8-Br-cAMPS (inhibitor of RII), or 10 μM H89 (inhibitor of CAT) in the presence of 1 μM, 10 μM or 20 μM forskolin. (A) FRET ratios. Forskolin-mediated dissociation of PKA subunits is prevented by Rp-8-Br-cAMPS (inhibitor of RII), whereas H89 (inhibitor of CAT) did not prevent forskolin-induced PKA subunit dissociation. (B) CBF. H-89 significantly blocks CBF increases, whereas Rp-8-Br-cAMPS allows CBF increases to only 15% above baseline at all forskolin concentrations tested. This partial effect of Rp-Br-cAMPS is likely due to an estimated 70% inhibition of PKA dissociation at the used concentration; the remaining activation of PKA cannot be discerned with FRET but still activates CBF to a certain degree. *$P$<0.05, comparing groups indicated by brackets.

seconds, 984.7±40.0 seconds vs 699.0±67.5 seconds and 1082.3±57.3 seconds vs 697.2±56.7 seconds for 1 μM, 10 μM and 20 μM forskolin, respectively. All times for the CBF decrease were significantly longer than the times for the FRET ratio decrease ($P$<0.05). Thus, these data indicate that CBF is slower to return to baseline than cAMP levels or PKA activation.

The same was true in permeabilized cells when basolaterally perfused with 20 μM, 50 μM and 100 μM cAMP ($n$=7). The times for the CBF vs FRET ratio increase were statistically the same (215.7±10 seconds vs 178.7±16.6 seconds, 221.3±20.8 seconds vs 248.6±23.9 seconds and 206.0±16.6 vs 172.9±17.7 seconds, respectively, all $P$>0.05), whereas CBFs lagged behind FRET ratios to return to baseline (335.4±3.1 seconds vs 214.3±17.0 seconds, 514.3±48.6 seconds vs 269.4±25.3 seconds and 545.0±56.2 seconds vs 149.0±17.4 seconds, respectively, all $P$<0.05). These data again indicate that CBF is slower to return to baseline than cAMP levels and that this effect is made more discernible at a higher $[cAMP]_i$.

These data show that the kinetics of the increases of cAMP levels and CBFs are identical in response to apical forskolin in non-permeabilized cells or basolateral cAMP in permeabilized cells, whereas CBF returns from the maximal response to baseline more slowly than the levels of cAMP.

### Inhibition of the catalytic site of PKA

To show that the increase of CBF upon stimulation of forskolin is dependent on a PKA-dependent phosphorylation event, non-permeabilized cells were pre-incubated for 30 minutes with 10 μM H89, which inhibits PKA phosphorylation activity but not the disassociation of PKA catalytic and regulatory subunits (Davies et al., 2000). The same concentration of H89 was added to all perfusion solutions (Fig. 9). In cells treated with H89 ($n$=8), FRET ratios increased by 0.0013±0.0001 AU, 0.0038±0.0003 AU and 0.0051±0.0004 AU (all $P$<0.05). The changes were not significantly different from cells perfused with 1 μM, 10 μM and 20 μM forskolin without inhibitor (all $P$>0.05). CBF in cells treated with H89 had statistically indistinguishable CBF baselines, but the increase in CBF was only 3.5±1.1%, 5.9±1.9% and 4.6±2.4% above baseline in response to 1 μM, 10 μM and 20 μM forskolin, respectively (all $P$<0.05 compared with controls of non-inhibited cells; Fig. 9). Thus, H89 inhibited the forskolin-induced increase in CBF while not affecting FRET, consistent with its mechanism of action. Both forskolin and H89 were prepared in dimethyl sulfoxide (DMSO) but we have shown previously that the used concentrations of DMSO had no effects on CBF (Lieb et al., 2002).

### Inhibition of dissociation of PKA subunits

To show that FRET ratios and CBF are inhibited when the dissociation of PKA subunits is prevented (and thereby also the activation of PKA), non-permeabilized cells were pre-incubated for 30 minutes with 400 μM Rp-8-bromoadenosine-3′5′-cyclic monophosphorothioate (Rp-8-Br-cAMPS), a cAMP antagonist that blocks PKA subunit disassociation and, thus, activation of PKA (Gjertsen et al., 1995). The same concentration of Rp-8-Br-cAMPS was added to the basolateral perfusate during the experiment (Fig. 9). This concentration of Rp-8-Br-cAMPS is expected to block about 70% of PKA dissociation (Gjertsen et al., 1995). In cells treated with Rp-8-Br-cAMPS ($n$=8), FRET ratios increased by only 0.0002±0.0001 AU, 0.0004±0.0003 AU and 0.0018±0.0004 AU (all $P$<0.05 compared to control) in cells treated with 1 μM, 10 μM and 20 μM forskolin, respectively. Cells treated with Rp-8-Br-cAMPS had statistically indistinguishable CBF baselines compared with control cells not exposed to inhibitor, whereas CBF increased by 15.4±2.8%, 17.6±4.5% and 16.7±2.8% above baseline in response to 1 μM, 10 μM and 20 μM forskolin, respectively ($P$>0.05 to control with 1 μM forskolin; $P$<0.05 to controls with 10 μM and 20 μM forskolin; Fig. 9). Thus, Rp-8-Br-cAMPS inhibited the forskolin-induced increase in CBF at the two higher forskolin concentrations while inhibiting the increases in FRET ratio at all forskolin concentrations. Since Rp-8-Br-cAMPS at the concentration used here is not expected to completely block the dissociation of PKA (Gjertsen et al., 1995), the initial increase in CBF upon application of 1 μM forskolin was possibly mediated by some PKA dissociation within cilia that had little effect on the overall cellular change in FRET ratios measured by our method.

## Discussion

cAMP is an important regulator of CBF. In ciliated mammalian cells, it has been shown that elevated cAMP concentrations lead to an increase in CBF (Di Benedetto et al., 1991; Ma et

al., 2002; Morse et al., 2001; Smith et al., 1996; Tamaoki et al., 1989b; Wyatt et al., 2005; Wyatt et al., 1998). Adrenergic drugs, known to increase CBF (Sanderson and Dirksen, 1989; Wyatt et al., 1998; Yang et al., 1996), also increase intracellular cAMP levels. Even though adenosine may inhibit CBF through A1 receptor activation and subsequent adenylyl cyclase inhibition in rabbit epithelia (Tamaoki et al., 1989a), adenosine has been reported to stimulate human airway CBF via A2b receptors through cAMP production (Morse et al., 2001).

Detailed, high-resolution information is available on how changes in the concentration of intracellular $Ca^{2+}$, another important regulator of mammalian CBF, are kinetically linked to changes in CBF (e.g. Lansley and Sanderson, 1999; Salathe and Bookman, 1999; Zhang and Sanderson, 2003). However, no previous information is available on the kinetics relationship of changes in cAMP and CBF. In general, there are only two means currently available to measure changes in $[cAMP]_i$ in any cell: using cAMP-gated channels to measure $[cAMP]_i$ changes near the plasma membrane (Rich et al., 2001), or using FRET with fluorescently tagged subunits of PKA (Adams et al., 1991; Zaccolo et al., 2000; Zaccolo and Pozzan, 2002) or the fluorescently tagged single-chain Epac (DiPilato et al., 2004; Nikolaev et al., 2004) to measure activation and deactivation of cAMP targets. All these methods require either recombinant expression of proteins or injection of labeled proteins into cells. Neither of these requirements is easily accomplished with ciliated cells. Injection attempts in our hands invariably lead to ciliostasis. In addition, recombinant expression of proteins in fully differentiated ciliated cells is difficult because these cells have developed highly effective ways to prevent transfections with plasmids or infections with viruses (e.g. Pickles, 2004). Non-differentiated airway epithelial cells, however, are more easily infected and exogenous protein expression is maintained in differentiated cells after lentiviruses infection with appropriate promoters. We show here that this strategy is successful.

Our results also demonstrate that the choice of promoter is crucial. The importance of using a promoter that is active in ciliated cells is supported by a recent publication showing that GFP can be expressed in these cells after infection of isolated fetal airway epithelial cells with a lentivirus using the human phosphoglycerate kinase promoter (Castillon et al., 2004). To exclude expression in non-ciliated cells, we chose the foxj1 promoter, which is ciliated-cell-specific (Ostrowski et al., 2003; You et al., 2004).

Using fluorescently tagged PKA subunits and determining their association or dissociation with FRET actually measures the activation and de-activation of PKA, a process that depends on $[cAMP]_i$, but does not strictly measure $[cAMP]_i$. In fact, this method seems less sensitive and kinetically less responsive to changes in $[cAMP]_i$, especially at lower concentrations, compared with measurements with cAMP-gated channels at the plasma membrane (Rich and Karpen, 2002) or with fluorescently tagged Epac (DiPilato et al., 2004; Nikolaev et al., 2004). However, CBF is regulated by axonemal PKA activity. Thus, measuring the activation and de-activation of PKA is a more direct way of evaluating how cAMP regulates CBF via PKA. In support of this notion, the rate of increases in both FRET ratios and CBF grew with increasing cAMP concentrations. Thus, it is conceivable that the actual $[cAMP]_i$ concentration increases more rapidly, but that the resulting activation of PKA is slower at low $[cAMP]_I$, as shown previously by comparison of the PKA and Epac FRET reporters of cAMP (Nikolaev et al., 2004). That PKA activation is important for CBF is reflected by the fact that the slower rate of PKA activation at lower $[cAMP]_i$ corresponds to a slower overall CBF activation. Using the new Epac-based FRET reporters to measure changes in $[cAMP]_i$ in ciliated cells might be able to answer some of these questions in the future.

When using permeabilized cells, we attempted to calibrate the system by comparing forskolin responses with directly applied cAMP at different concentrations. Using this system, it still remains somewhat unclear what cAMP levels can be reached at the apical membrane, because cAMP is usually not able to freely diffuse throughout the cell because of phosphodiesterase activities. Notice, however, that permeabilization may have interrupted the activity of these phosphodiesterases because experiments using non-hydrolysable analogs of cAMP did not show any significant differences with respect to FRET ratios and CBF compared with regular cAMP (data not shown).

This study reveals that kinetically, PKA activation inside cells leads to a simultaneous increase in CBF (within the measurement resolution of 10 seconds). In some measurements, CBF rose initially faster than the FRET ratio, possibly indicating that the sensitivity of the FRET signal is low (as discussed above) and thus lags the CBF rise, or only a small amount of PKA dissociation is necessary to produce the initial rise in CBF. When PKA activation is blocked by inhibiting the PKA catalytic site or preventing PKA dissociation, no CBF increase is seen (with the exception of the 1 μM forskolin data when using the regulatory domain blocker Rp-8-Br-cAMPS, which is probably due to incomplete inhibition of PKA dissociation).

Whereas cAMP via activation of PKA increases ciliary movement, CBF is slower to return to baseline than PKA activity. It is possible that whole-cell FRET ratios are misleading with respect to the changes in the cilium and that cAMP concentrations remain longer elevated in the cilium (but an isolated intraciliary signal cannot be detected with our methods). Another explanation could be that ciliary target proteins remain phosphorylated, even in the absence of PKA activity, and that phosphatase activity is required before CBF can return to baseline. A low but constant level of phosphatase activity could explain the slower CBF decrease when PKA activity declines briskly, likely concurrent with the reported rapid cAMP hydrolysis (Nikolaev et al., 2005). The kinetics of this CBF decay towards baseline could therefore provide insight into ciliary phosphatase activities, which could also be regulated. The result of prolonged CBF increases after increased termination of PKA activity confirms an older, indirect suggestion for the need of phosphatases to revert cAMP-mediated CBF increases back to baseline; there, PKA inhibitors could not return CBF immediately to baseline when applied 2 minutes after stimulation of PKA (Lieb et al., 2002). In addition, the presence of phosphatases in the mammalian axoneme has been recently demonstrated (Gertsberg et al., 2004).

We have previously reported that prolonged CBF stimulation after transient increases of intracellular $Ca^{2+}$ $[Ca^{2+}]_i$ are due to cAMP production (Lieb et al., 2002). Thus, the question arises whether increases in cAMP trigger $[Ca^{2+}]_i$ increases in these

cells. However, we could not detect $[Ca^{2+}]_i$ increases upon forskolin stimulation in human airway epithelial cells (Lieb et al., 2002). Thus, elevated $[Ca^{2+}]_i$ does not explain the prolonged CBF stimulation after a transient cAMP increase.

Finally, it was important that the recombinant, fluorescently tagged PKA subunits were localized to cilia (Fig. 4), the location of naturally expressed CAT and RII subunits of PKA (Kultgen et al., 2002). We were, however, concerned that overexpression of PKA could lead to an alteration in CBF regulation. This is not likely because cells not infected with lentiviruses show the same CBF responses to forskolin in a non-permeabilized state and to forskolin or cAMP in a permeabilized state as cells that overexpress recombinant PKA.

## Materials and Methods

### Chemicals and solutions

LHC medium, trace elements, and stock 4 and 11 were from Biosource International (Rockville, MD); Gentamicin from Gibco BRL Laboratories (Grand Island, NY); fetal bovine serum was from HyClone (Logen, UT). All other reagents were from Sigma Chemicals (St Louis, MO) unless otherwise indicated. The composition of solutions used for apical and basolateral perfusion is shown in Table 1. The basolateral solution used for experiments with permeabilized cells contained ATP so that cilia were able to beat.

### 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 with local IRB-approved written consents conforming to the standards set by the Declaration of Helsinki. From these lungs, airway epithelial cells were isolated, de-differentiated through expansion and re-differentiated at an air-liquid interface (ALI) on 24-mm T-clear filters (Costar Corning, Corning, NY) as described (e.g. Bernacki et al., 1999; Fragoso et al., 2004; Nlend et al., 2002). NCI-H292 cells were obtained from American Type Culture Collection (ATCC) and cultured accordingly.

### Production of pseudo-typed lentivirus vectors and infection of airway epithelial cells or NCI-H292 cells

A gene-transfer method using a third-generation, propagation-deficient, HIV-pseudotyped lentivirus was developed to infect NCI H292 and undifferentiated airway epithelial cells. For airway cells, the goal was to obtain expression of two tagged subunits of PKA at the end of differentiation into a ciliated phenotype. Recombinant lentiviruses were constructed using the pRRLsinPPT.CMV.MCS.Wpre vector (De Palma and Naldini, 2002). For the initial constructs, genes encoding the catalytic PKA subunit CAT and the regulatory PKA subunit RII, fused to the fluorescent proteins YFP and CFP, respectively (Zaccolo and Pozzan, 2002) were cloned into the multiple cloning site downstream of the CMV promoter to produce pRRLsinPPT.CMV.CATYFP and pRRLsinPPT.CMV.RIICFP, respectively.

For better expression in ciliated cells, the CMV promoter was replaced by the ciliated-cell-specific *foxj1* promoter (Ostrowski et al., 2003). A *foxj1* promoter fragment with a *Nde*I and *Xba*I restriction site at the 5′ and 3′ end, respectively, was prepared by PCR amplification of a 1 kb cloned genomic fragment using *foxj1*-specific primers with the restrictions sites appended. This fragment was directionally cloned into *Nde*I and *Xba*I sites of the original RII-CFP and CAT-YFP lentiviral expression plasmids, deleting about 300 bp from the 3′ end of the CMV promoter, thereby rendering it inactive. Constructs were confirmed by sequencing.

Lentiviruses were prepared by co-transfecting HEK 293T cells with vector and packaging DNAs plasmids, pMDLg/pRRE#54', pRSV-Rev and pMDLgVSVG, using calcium phosphate co-precipitation. Virus-containing medium was collected every 24 hours for 3 days, cells were removed by centrifugation at 2500 ***g*** for 5 minutes. The medium was then passed through a 0.45-μm filter. The filtrate was used directly for infection, or was concentrated by ultracentrifugation or polyethylene glycol (11%) precipitation. The quality of virus production was measured by p24 ELISAs (PerkinElmer, Wellesley, MA).

Undifferentiated airway epithelial cells (up to the second passage) were plated onto collagen-coated T-col filters (0.4 μm) at 500,000 cells per 24-mm filter in bronchial ephitelial medium (BEGM). The day of infection, 40 ng [estimated multiplicity of infection (MOI): 0.24] of each viral construct were mixed in BEGM containing 6 μg/ml polybrene (final concentration), added to the cells and incubated overnight in 37°C in 5% $CO_2$. The following day, virus was discarded and medium was changed to ALI medium (e.g. Bernacki et al., 1997; Fragoso et al., 2004; Nlend et al., 2002), top and bottom. After cells reached confluence, apical media were removed to establish the ALI. NCI-H292 cells were plated on cover slips and infected according to the same protocol but with a MOI of 0.8. Infected cells were monitored by p24 ELISA for any unexpected virus shedding; shedding was never detected, thus ruling out culture contamination with replicating virus.

### FRET and CBF measurements in airway epithelial cells

NCI-H292 cells grown on collagen-coated glass cover slips were mounted onto the stage of an upright Nikon E600fn microscope in a Warner Instrument RC-25F perfusion chamber (Warner Instruments, Hamden, CT) with a 150 μl working volume and imaged with a 60× water immersion objective. ALI cultures were placed in a customized chamber allowing independent perfusion of the basolateral and apical compartment, and mounted at room temperature onto the stage of the Nikon Eclipse microscope. If required, airway epithelial cells were permeabilized as described before (Sutto et al., 2004).

CFP excitation (20-nm-wide filter-centered on 436 nm, Chroma) was controlled by Metafluor™ software (Molecular Devices Corporation, Downingtown, PA) via a DG4 rapid-wavelength switcher (Sutter Instruments, Novato, CA). Cells were imaged with a CoolSnap Hq cooled CCD camera (Roper Scientific, Tucson, AZ), attached to the microscope using a Dual-View (Optical Insights, Tucson, AZ). The Dual-View duplicates the center 50% of the image and reflects the two images side-by-side onto the CCD chip, thereby allowing simultaneous visualization and recording of the same cell at two different emission wavelengths. The Metafluor™ FRET module was used to measure CFP/YFP ratios.

CBF was estimated according to published methods with the apical surface 'submerged' (Salathe and Bookman, 1999) using infrared differential interference contrast (IR-DIC) video microscopy. Using a Nikon dual-image module and guiding the infrared signal to a Sony XC-7500 CCD camera while sending all fluorescence signals (<600 nm) to the Dual-View in front of the cooled CCD camera, CBF and FRET of the same cell were measured simultaneously. In a field, any ciliated cell expressing both fusion proteins was selected for simultaneous measurement of CBF and FRET at random and the selection was not guided by pre-determined fluorescence levels. The maximum number of cells in a field measured was three, limited by our ability to simultaneously track CBF in different cells. Results were compared by ANOVA and, if significantly different, by the Tuckey Kramer honestly significant difference test for differences between groups.

### Immunohistochemistry

Differentiated normal human airway epithelial cells grown on 0.4 μm 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. Cells were blocked with 2% bovine serum albumin. Cilia were visualized with anti-acetylated tubulin antibody (1:500; Sigma) using an Alexa Fluor-555 labeled goat anti-mouse antibody as secondary label (1:2000; Molecular Probes). If desired, DAPI was added before mounting to stain nuclei.

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