Asymmetric interactions between phosphorylation pathways regulating ciliary beat frequency in human nasal respiratory epithelium *in vitro*

R. P. Smith, R. Shellard*, D. P. Dhillon, J. Winter and A. Mehta*†

*Departments of *Child Health (Centre for Research into Human Development), Ninewells Hospital and Medical School and Respiratory Medicine, King's Cross Hospital, Dundee DD1 9SY, UK*

1. The effects of the sequential stimulation of ciliary beat frequency (CBF) via two different phosphorylation cascades (dependent on protein kinase A (PKA) and calmodulin, respectively) were determined using video microscopy applied to a perfused preparation of human nasal respiratory epithelium *in vitro*. Dibutyryl cyclic AMP (db-cAMP) (10^{-3} M) was used to stimulate PKA and the calcium ionophore 4-Br-A23187 (10^{-5} M) was used to stimulate calmodulin-dependent phosphorylation.

2. Perfusion with db-cAMP (10^{-3} M) alone showed an early rise in CBF (15·0 ± 4%, mean ± S.E.M., $P < 0·05$) by 10 min which remained elevated for 35 min; in contrast, the highest CBF response to 4-Br-A23187 (10^{-5} M) alone was not achieved until 35 min (16·1 ± 1·8%, $P < 0·05$).

3. When a db-cAMP stimulus was applied to cells which had been pre-incubated with 4-Br-A23187 for 30 min, a further rise in CBF (maximal at 20 min, 14·3 ± 2%, $P < 0·05$) was observed. Reversing the sequence of perfusions, cells pre-incubated with db-cAMP showed no further rise in response to stimulation with 4-Br-A23187.

4. We hypothesized that PKA inhibited the response to the 4-Br-A23187. This notion was supported by the restoration of the CBF response (22·8 ± 4%, $P < 0·05$) to 4-Br-A23187 when the cells were pre-incubated with the protein kinase inhibitor 1-(5-isoquinolinyl-sulphonyl)-2-methylpiperazine (10^{-3} M), before the sequential perfusions with db-cAMP and 4-Br-A23187. We conclude that the A23187-dependent pathway, which regulates intrinsic CBF, is inhibited by db-cAMP but not vice versa.

Mucociliary clearance is the defence mechanism by which inhaled particles and local cellular debris are removed from the lung by beating cilia, which expel this mucus-entrapped debris towards the oropharynx (Sleigh, Blake & Liron, 1988). This mucus floats on a periciliary fluid elaborated from the epithelium, with the efficiency of clearance dependent on an optimal interaction between the specialized 'claws' on the apices of respiratory cilia and the underside of the mucus layer. Although the mechanisms which integrate mucus release (Ramnarine, Hirayama, Barnes & Rogers, 1994) with mucus hydration and ciliary activity are incompletely understood, there is overwhelming evidence that protein phosphorylation regulates two key components of mucociliary clearance: the ciliary beat frequency (CBF) and the ion transport which results in fluid absorption/secretion (Sanderson & Dirksen, 1989; Liedtke, 1992; Ismailov & Benos, 1995). Additionally, cell calcium (± calmodulin) has the potential to integrate all aspects of the mucociliary apparatus: mucus secretion, epithelial fluid elaboration and CBF (Baker, Hilegass, Holden & Smith, 1977; Liedtke, 1992; Salathe, Pratt & Wanner, 1993; Ramnarine *et al.* 1994; Smith, Shellard, Di Benedetto, Magnus & Mehta, 1996).

Theoretical models of mucociliary clearance (Satir, Barkalow & Hamasaki, 1993) suggest that the driving force which expels mucus from the lung is dependent on the square of the CBF. Thus small increments in CBF may exert disproportionately large effects on mucus clearance. The *in vivo* relationship between clearance and CBF is supported by experiments conducted in the human nose. At this site there is a linear relationship between the log of the *in vivo* saccharine clearance time (the time taken for a particle to travel from the inferior turbinate to pharynx) and the mean

† To whom correspondence should be addressed.

in vitro nasal CBF measured from cotemporaneous turbinate biopsies (Duchateau, Graamans, Zuidema & Merkus, 1985). This finding makes the nasal epithelium a relevant model for further study of the physiological regulation of CBF. Our previous *in vitro* experiments (Di Benedetto, Magnus, Gray & Mehta, 1991*a*; Di Benedetto, Manara-Shediac & Mehta, 1991*b*) revealed that human nasal CBF is differentially stimulated via apparently independent PKA- and calcium–calmodulin-dependent phosphorylation pathways. These pathways differed in two key respects. (1) We found that the CBF response to stimulation with dibutyryl cAMP (db-cAMP) (10^{-3} M) was of rapid onset (at plateau by 10 min and sustained for 35 min). In contrast, despite a sevenfold rise in intracellular calcium to the submicromolar range within 10 min of exposure to the calcium ionophore 4-Br-A23187 (10^{-5} M), the onset of the resultant CBF response was relatively delayed (plateau reached by 35 min). (2) The intrinsic CBF (Di Benedetto & Mehta, 1990) declined by 10% when calmodulin-dependent phosphorylation was inhibited (Di Benedetto *et al.* 1991*a*), whereas inhibition of PKA/protein kinase C (PKC) had no effect on intrinsic ciliary activity. These studies, combined with evidence from animal experiments (Tamaoki *et al.* 1991), led us to the notion that intrinsic CBF was regulated differently from stimulated CBF. The present study tests the limits of our hypothesis.

We have used a new video technique for the determination of CBF (Rusznak, Devalia, Lozewicz & Davies, 1994) to study the net effects of sequentially activating the PKA- and the calcium–calmodulin-dependent phosphorylation cascades which regulate CBF in human nasal respiratory epithelium *in vitro*. We test the hypothesis that these pathways interact with each other. Firstly, we referenced the new video-based technique against the individual, photometrically determined ciliary responses to 4-Br-A23187 (10^{-5} M) and db-cAMP (10^{-3} M) (Di Benedetto *et al.* 1991*a*, *b*, respectively). Secondly, we stimulated an initial rise in CBF using 4-Br-A23187 (to raise intracellular calcium) and then measured the CBF response to a subsequent stimulus with db-cAMP (to stimulate PKA). We then reversed the protocol by applying an initial db-cAMP stimulus followed by 4-Br-A23187. We describe an asymmetric interaction between these pathways and show that whereas PKA abolishes the CBF response to 4-Br-A23187, this calcium ionophore cannot abolish the db-cAMP-dependent ciliary response.

METHODS

Subject selection

Respiratory epithelium was obtained immediately after the induction of general anaesthesia from non-asthmatic, non-atopic patients undergoing routine operations unrelated to nasal disease (age range, 9–45 years). These patients had no respiratory infections for 1 month prior to operation. We and others have previously shown that smoking, local/general anaesthesia, subject age or cell storage in Medium 199 at 4 °C have no effect on either intrinsic CBF or its response to db-cAMP *in vitro* (Di Benedetto *et al.* 1991*b*). Approval for the study was given by the Tayside Committee on Medical Research Ethics and informed written consent was obtained from the patients.

Sample collection

Strips of ciliated epithelium were obtained with a cytology brush from the inferior turbinate using a technique described by Rutland & Cole (1980). Cellular material adherent to the brush was dislodged by brisk agitation in Eppendorf tubes containing 1 ml of tissue culture medium (Medium 199; Flow, Rickmansworth, UK). All experiments were performed within 4 h of collection and the samples were kept in Medium 199 at 4 °C for this period.

Measurement of ciliary beat frequency

Video technique. CBF was measured at room temperature (23 ± 1 °C) using a video-based, Hoffman contrast technique (Brian Reece Scientific, Newbury, Berks, UK) as recently described by our group and others (Smith *et al.* 1996; Rusnak *et al.* 1994). There are four main stages, which are described briefly here. (a) The technique permits the selection of any desired area of the video field (e.g. adjacent to the luminal border of strips of ciliated cells) either via a video-camera (linked to a television monitor) or via the eye-piece of an inverted microscope. (b) It samples contrast changes fifty times per second over any desired point on the microscope/video field. The change in contrast is induced by the sweep of the cilia as light is passed downward through the sample chamber. The 50 Hz sampling rate exceeds the typical range of observed ciliary frequencies (4·5–10 Hz) by a factor of 5, thus eliminating artefacts in the estimation of frequency induced by the Nyquist limit (sampling must be at least twice the frequency being measured). (c) This contrast signal is digitized and the individual ciliary waveforms are displayed in real time to check signal quality. (d) A mean frequency is calculated from the number of peaks in a 2 s interval. This process enables the reproducible measurement of changes in CBF provided they exceed 5% of baseline when combined with a rigid adherence to our protocol for cell border selection (which provides stable baseline signals, see below).

Cell perfusion. The chamber design and blinded perfusion protocol have been described in detail elsewhere (Di Benedetto *et al.* 1991*a*; Smith *et al.* 1996). Briefly, an aliquot from freshly brushed strips of ciliated cells was transferred in Medium 199 to a glass-walled perfusion chamber with an internal volume of 0·35 ml. The chamber was connected to a gravity-fed perfusion system which delivered 0·25 ml min^{-1} and gave a 95% wash-out of the fluid in the chamber in 10 min. In order to eliminate the increase in CBF induced by perfusion (Di Benedetto, Lopez-Vidriero & Clarke, 1987), all measurements used for calculation of the results were taken in the absence of flow. The pH of all solutions used was between 7·2 and 7·4, a range known not to affect bronchial ciliary activity (Luk & Dulfano, 1983). The extracellular Ca^{2+} concentration was 1·8 mM, which is within the range known not to affect CBF in rabbit tracheal explants (Girard & Kennedy, 1986).

Cell selection. Ciliated strips formed by a sheet of ten or more cells attached to a basement membrane were selected at random and CBF measurements were taken from the same point adjacent to the ciliated cell border throughout each experiment by an observer who was unaware of the nature of the perfusates. A ciliated strip was rejected unless its CBF signal was (a) sinusoidal (with no M-shaped complexes induced by inclusion of the reverse ciliary sweep to the resting position for the next ciliary cycle) and (b) stable, i.e. CBF returned to its original waveform within 30 s of transient changes in flow induced by repeated manual compression of the tubing from the reservoir of medium.

Study design

The protocol involved a maximum of four sequential perfusions. (1) There was an initial 30 min period of equilibration (in either Medium 199 alone or with the kinase inhibitor H-7) to allow the cell strips to adhere to the glass walls of the chamber and to establish that the cells were beating synchronously (but no CBF measurements were made at this time). (2) A subsequent perfusion was applied with 'blind' medium containing one or more agonist/inhibitor or an appropriate control medium (Medium 199 plus vehicle alone; see below) followed by a further incubation for 30 min. A cell strip with a stable CBF signal was then selected. In some experiments cells needed to be allowed enough time to equilibrate with more than one agent (e.g. the sequential perfusion experiments) and in order to avoid wash-out artefacts, the concentration of the agent under investigation was maintained throughout the period of study. (3) Baseline CBF measurements were then recorded from the chosen cell strip at 10, 20 and 30 min. 'Baseline CBF' was defined as the mean of these three readings, and for all subsequent measurements this baseline was defined as 100%. (4) A second blinded 10 min perfusion was then applied containing either the test substance or control medium and CBF was recorded at 5 min intervals for a further 40 min.

We have previously shown that human nasal respiratory cells are able to tolerate the length of perfusion used in these experiments without deleterious effects on CBF (Di Benedetto *et al.* 1991*a*,*b*; Smith *et al.* 1996), provided precautions are in place to minimize any light-induced rise in chamber temperature. The microscope was therefore switched off between readings and its stage was cooled with a fan. Chamber temperature was measured continuously with a thermocouple and experiments were rejected if the rise in temperature exceeded 1 °C. All substances were studied at room temperature using a randomized, blinded protocol which included a parallel study of an appropriate control solution for each experiment. For calculation of the results within each limb of an experiment, the mean CBF results quoted were determined from the mean of the individual CBF values of ten cell borders located along each strip (unless otherwise indicated), each CBF value having been recorded from a different subject. The vehicle for each experiment was Medium 199 and the section below indicates the protocol for perfusion in each experiment.

Perfusion A. Validation study. Following a 30 min period to allow the cells to adhere to the glass chamber, baseline CBF was determined (see above). The rise in CBF following perfusion with db-cAMP (10^{-3} M), 4-Br-A23187 (10^{-5} M) or Medium 199 alone was determined.

Perfusion B. The effects of sequential activation on the terminal CBF response. The initial 30 min period of cell adherence was undertaken in Medium 199 containing either: db-cAMP (second perfusion, 4-Br-A23187) or 4-Br-A23187 (second perfusion, db-cAMP). In order to ensure temperature stability (see above) after the second perfusion, it was not possible to record baseline CBF during the 30 min period of pre-incubation with either db-cAMP, 4-Br-A23187 or medium alone. Instead, we simply allowed sufficient pre-incubation time for a plateau in the CBF response to have occurred before the second infusion was applied.

In separate experiments, we inhibited the effect of db-cAMP on CBF by adding the PKA/C inhibitor H-7 (1-(5-isoquinolinylsulphonyl)-2-methylpiperazine, 10^{-3} M) (Hidaka, Inagaki, Kawamoto & Sasaki, 1984) to the medium both before and during the incubation with db-cAMP; following this period either 4-Br-A23187 or db-cAMP + H-7 was added to the second infusion as above.

Chemical reagents

All reagents were of analytical grade and purchased from Sigma with the exception of Medium 199 (Flow, Rickmansworth, UK).

Statistical analysis

The randomization protocol for the selection of the cell border resulted in a variation in mean baseline CBF between experiments of approximately 1 Hz. We have previously analysed the change in CBF in response to db-cAMP and 4-Br-A23187 and found statistically significant responses to both agents (compared with their respective baselines). The significance of the response was independent of the mode of analysis, i.e. we obtained the same significance following analysis of the change in the raw frequencies (typical range, 5–9 Hz) or the percentage changes from baseline (Di Benedetto *et al.* 1991*a*,*b*). Here, the observed CBF responses to the agonists were expressed as the percentage changes from baseline CBF and the mean (± S.E.M.) frequencies are given in the figure legend. Two types of analysis of variance (ANOVA) were used to compare the CBF responses to different infusions: either simple ANOVA (comparison of individual time points) or ANOVA with multiple range testing (comparison between time courses for each limb). For paired data (baseline *versus* post-infusion CBF), Student's paired t test was used. For all tests, significance was accepted when $P < 0{\cdot}05$.

RESULTS

Perfusion A. Validation of the video technique with single stimuli

Our previous data on the differences between the time course of the CBF responses to db-cAMP and 4-Br-A23187 were obtained using a cumbersome photometric technique (Di Benedetto *et al.* 1991*a*,*b*). Comparison of the results (shown in the first perfusion period of Fig. 1) with our previously published data (Di Benedetto *et al.* 1991*a*,*b*) shows that db-cAMP (10^{-3} M) and 4-Br-A23187 (10^{-5} M) produced their respective, expected increments in CBF with respect to both timing and magnitude using the new video contrast technique. In contrast, the control infusion with Medium 199 alone (Fig. 1) or with H-7 (data not shown) showed no significant rise in CBF.

Dibutyryl cyclic AMP produced an early rise in CBF ($15{\cdot}3 \pm 4{\cdot}1\%$ at 10 min; $P < 0{\cdot}05$ for this time point and thereafter) which was sustained throughout the period of measurement (40 min). Furthermore, the calcium ionophore 4-Br-A23187 induced a relatively delayed rise in CBF (an early rise of $9{\cdot}0 \pm 2{\cdot}8\%$ at end of 10 min infusion period; $P < 0{\cdot}05$, compared with baseline) with the response reaching a plateau at 35 min ($16{\cdot}1 \pm 1{\cdot}8\%$; $P < 0{\cdot}05$). We have previously shown that the plateau responses to both agents were sustained for a further 30 min. At 10 and 15 min post-infusion, the percentage rise in CBF after db-cAMP application was significantly greater than with 4-Br-A23187 ($P < 0{\cdot}001$), but there was no significant difference in the magnitude of the response to the two agents beyond 15 min. At all time points, both agents showed a highly significant difference ($P < 0{\cdot}001$) compared with the control infusion with either Medium 199 alone or Medium 199 + H-7 (data not shown).

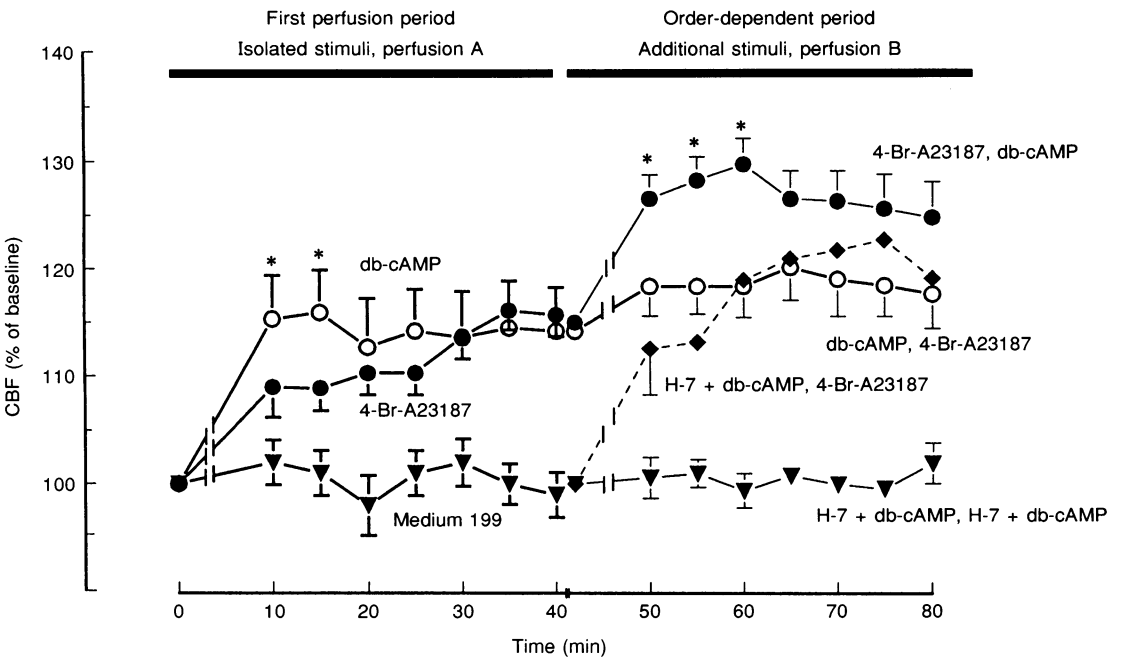

Figure 1. Protein kinase A inhibits calmodulin-dependent control of ciliary beat frequency

The bars divide the figure into 2 perfusion periods. The filled bar A shows the time of the first perfusion; ordinate shows the percentage change in ciliary beat frequency (CBF) with 3 different stimuli given alone. During the second perfusion (filled bar B), the ordinate shows the subsequent CBF response to 4 different sequential perfusions; the (comma-delimited) order of perfusion is shown adjacent to each dataset. The abscissa shows experimental time and the break at 40 min indicates that the perfusions A and B were carried out in different sets of experiments. Each data point represents the mean percentage change in CBF (means ± S.E.M., $n = 10$ unless otherwise indicated). The break in the graph between 0–10 and 40–50 min reflects the absence of data during chamber filling. The first perfusion (A) shows the effect of control Medium 199 alone (▼), or medium containing either 10^{-3} M db-cAMP (○) or 10^{-5} M 4-Br-A23187 (●) on the CBF of human nasal epithelium when compared with their respective baseline CBF values (baseline is shown as 100% on the ordinate). The mean CBF before the perfusion of the chamber with one of the above solutions was 7·0 ± 0·4, 7·1 ± 0·3 and 7·7 ± 0·5 Hz, respectively. When compared with control, both db-cAMP (15·3 ± 4·1%) and 4-Br-A23187 (16·1 ± 1·8%) elevated CBF significantly above baseline ($P < 0·001$, at all time points between 10 and 40 min). The rise in CBF at 10 and 15 min with db-cAMP was significantly greater (*) than with 4-Br-A23187 ($P < 0·01$). The 4-Br-A23187 response reached its zenith 20 min after the maximal db-cAMP response. There was no significant difference between the percentage changes in CBF in response to db-cAMP and 4-Br-A23187 by 30 min. The second perfusion (B) with additional stimuli showed the effect of the order of perfusion on the CBF response using cells already beating at ~15% above their original baseline in A (db-cAMP-stimulated cells, ○; 4-Br-A23187-stimulated cells, ●). This second perfusion (B) contained one of the following: db-cAMP, 4-Br-A23187 (○); 4-Br-A23187, db-cAMP (●); H-7 + db-cAMP, 4-Br-A23187 (◆); or H-7 + db-cAMP alone (▼). Where no error bar is shown it lies within the confines of the symbol except for the H-7 + db-cAMP limb, which has been omitted for clarity (magnitude illustrated for the 50 min time point only). When compared with the baseline (at 4-Br-A23187-induced plateau), there was a significant further increment in CBF (*) between 50 and 60 min, when db-cAMP followed 4-Br-A23187 (●). In contrast, when the order of perfusion was reversed, no significant rise in CBF was seen in cells first exposed to db-cAMP and then stimulated with 4-Br-A23187 (○). This CBF response was restored when H-7 (10^{-3} M) was present before and during the incubation with db-cAMP leaving the order of perfusion unchanged (◆). The H-7 + db-cAMP combination in the absence of 4-Br-A23187 showed no significant rise in CBF above baseline (▼, $n = 4$). Analysis of the three curves by ANOVA showed significantly enhanced responses to db-cAMP in infusion B (●, $P < 0·0001$) when compared with 4-Br-A23187 (○) or H-7 + db-cAMP, 4-Br-A23187 (◆).

Perfusion B. The effects of sequential activation on the terminal CBF response

(i) In cells which had previously been exposed to 4-Br-A23187 (10^{-5} M), a subsequent stimulus with db-cAMP induced a further rise in CBF of $14{\cdot}8 \pm 2{\cdot}4\%$ ($P < 0{\cdot}05$ when compared with the post-ionophore CBF). The rate of rise of CBF differed from that expected with db-cAMP alone because the peak response was delayed by 10 min and was not sustained (i.e. see uppermost response following infusion B in Fig. 1). (ii) Following a reversal of the order of stimulation, no response was observed to the second stimulus. At a time when a plateau in the CBF response to the first infusion with db-cAMP should have been present (i.e. 30 min post-infusion A), there was no further rise in CBF with 4-Br-A23187 (expected rise *ca* 15%). The observed rise in CBF of $5{\cdot}2 \pm 3{\cdot}1\%$ at 25 min from the start of perfusion B was not statistically different from 'baseline' CBF (i.e. that in the presence of an elevated CBF via db-cAMP). Comparison of the time course of the responses to the two stimuli showed that the db-cAMP-induced rise in CBF was significantly higher than the 4-Br-A23187-induced rise for the first 20 min post-infusion B ($P < 0{\cdot}001$). Thereafter, the decay in the db-cAMP-induced response (65–80 min) eliminated any difference between the two stimuli.

We tested the hypothesis that db-cAMP, acting via PKA, was inhibiting the calmodulin-dependent cascade by which 4-Br-A23187 normally acts (see the lower two graphs in the order-dependent perfusion period of Fig. 1). We have previously shown that the protein kinase antagonist H-7 (10^{-3} M), when pre-incubated for 30 min, abolishes the PKA-modulated stimulatory effects of db-cAMP on CBF but has no effect on intrinsic CBF (Di Benedetto *et al.* 1991*b*) for up to 90 min post-perfusion (Shellard, 1994). Here, we find that when H-7 was first pre-incubated (30 min) and then co-infused with db-cAMP (10^{-3} M) during perfusion A, the additional presence of 4-Br-A23187 in perfusion B restored the response. When 4-Br-A23187 was omitted in the control limb, no rise in CBF was observed. These data are consistent with an inhibitory effect of db-cAMP on the calmodulin pathway mediated by PKA-dependent protein phosphorylation. In the presence of db-cAMP + H-7, the rise in terminal CBF induced by 4-Br-A23187 ($22{\cdot}8 \pm 4\%$ at 35 min, Fig. 2) was higher that the plateau achieved with the sequential infusion of db-cAMP + 4-Br-A23187 but just failed to reach significance by ANOVA ($P = 0{\cdot}07$).

DISCUSSION

CBF and the interaction between PKA- and calcium–calmodulin-dependent pathways

Intracellular pathways communicate at many levels within signal transduction cascades: from the receptor (Bylund, 1992; Felder, 1995), receptor-linked effectors such as the adenyl cyclases (Iyengar, 1993), to G proteins and protein kinases (Taylor, 1990). Here we find that in human nasal cilia, the interaction which occurs between the PKA and calcium–calmodulin (CAM) pathways regulating CBF is dependent on the order of perfusion of db-cAMP and 4-Br-A23187. Specifically, when db-cAMP activates PKA at resting (nM) cell calcium, a subsequent 4-Br-A23187 stimulus fails to activate the CAM pathway. We cannot ascribe this failure of CAM stimulation to a PKA-induced rise in cell calcium because no such rise has been observed in human cells following a cAMP-mediated stimuli (Paradiso, Cheng & Boucher, 1991). Our data suggest that this failure to activate the CAM pathway is mediated by an inhibitory phosphorylation event because the CAM response to the calcium ionophore was restored when kinase(s) activity was blocked with H-7. Our data are also consistent with a partial inhibitory action between the CAM-dependent phosphorylation and the rise in CBF with db-cAMP. This is illustrated by the unsustained response to db-cAMP when CAM kinase was activated by 4-Br-A23187 in the first infusion. We have previously observed that CAM-dependent phosphorylation is necessary for about 10% of the intrinsic CBF (i.e. that observed without external stimuli other than Medium 199). Whether this residual CAM activity inhibits the CBF response to the basal cAMP produced within the cell in the absence of agonists is unknown.

Evidence that different pathways regulate intrinsic and stimulated CBF

Although not statistically significant, in the presence of H-7 and db-cAMP, the magnitude of the 4-Br-A23187-induced CAM response (*ca* +22%) was higher than we have ever observed with 4-Br-A23187 alone (*ca* 16% (present study), 16% (Di Benedetto *et al.* 1991*a*) and 18% (R. P. Smith & A. Mehta, unpublished observations). We considered the explanation that H-7 relieved some form of 'tonic, phosphorylation-dependent inhibition of intrinsic CBF' via the CAM pathway (e.g. via PKC), thus augmenting the stimulated CBF response to PKA. This explanation is unlikely to be correct because we and others have previously shown that H-7 has no effect on intrinsic CBF in human or sheep airway cells (respectively, Di Benedetto *et al.* 1991*b*; Kobayashi *et al.* 1992), suggesting that PKC-like kinases are not active in the unstimulated state. In addition, we have shown that activation of PKC with the phorbol ester 4β-phorbol 12-myristate, 13-acetate (PMA) does not change intrinsic CBF in human nasal cells (Di Benedetto *et al.* 1991*b*), suggesting that PKC alone cannot stimulate CBF. However, PMA-treated cells show a doubling of the magnitude of the response to db-cAMP (Shellard, 1994). Thus if a non-PKA, H-7-sensitive kinase such as PKC plays any role in the regulation of human nasal CBF, its effects are likely to be confined to the stimulated state.

What is the evidence for the proposal that intrinsic CBF is regulated differently from stimulated CBF? This notion is supported by data from cultured bovine bronchial epithelium (Jain, Rubinstein, Robbins, Leise & Sisson, 1993). These workers found that the effects of nitric oxide (NO) on

intrinsic and stimulated CBF differed as follows: firstly, inhibition of NO production in the absence of receptor stimulation did not alter intrinsic CBF; and secondly, when CBF was first elevated via either a β-receptor agonist or a calcium-linked stimulus, any subsequent inhibition of NO production resulted in an immediate cessation of the ciliary response irrespective of the second messenger released by the activated receptor. Interestingly, the magnitude of this rapid decline in CBF was different for the cAMP- and calcium-activated pathways. When a β-receptor was activated, the CBF increased to a new plateau but declined precipitately to below the pre-stimulation frequency following the loss of the NO drive. In contrast, when calcium-linked receptors had been activated, subsequent inhibition of NO drive merely induced a prompt return to (but not below) the pre-stimulation ciliary frequency. Based on our hypothesis that PKA inhibits the CAM-dependent pathway for the regulation of CBF, we offer the following explanation for their data. We propose that when bovine bronchial CBF was elevated by PKA, a parallel and silent inhibition of the CAM pathway was induced and was only unmasked by the elimination of the NO drive. Our model also explains why inhibition of the CAM drive did not occur when the calcium-linked receptors were activated, hence the return to (but not below) baseline CBF when NO production was inhibited. Although attractive, the known difficulties with cross-species comparisons (Kobayashi, Tamaoki, Sakai, Chiyotani & Takizawa, 1989; Kobayashi *et al.* 1992) require the validation of our model in human cells.

Molecular models for the interaction between PKA and the CAM pathway

Molecular models of ciliary regulation (Lansley, Sanderson & Dirksen, 1992) are centred around the proteins associated with the axoneme (Stephens & Prior, 1992) and provide biochemical paradigms for the data presented here from the human nose. For example, in ciliary membranes from *Paramecium* (Satir, Barkalow & Hamasaki, 1993), the sliding velocity of axonemal microtubules (and hence CBF) is critically dependent on the PKA-dependent phosphorylation of a 29 kDa phosphoprotein associated with the outer dynein arm. PKA is unable to phosphorylate this protein in the presence of high intracellular calcium, conditions which also fail to activate sliding. We have observed a phosphoprotein of a similar size in apical preparations of human nasal and ovine tracheal respiratory epithelium (Treharne, Marshall & Mehta, 1994; S. J. Banner & A. Mehta, unpublished observations) but have no data with respect to their function(s). Others have reported an equivalent protein in sheep tracheal ciliary axonemes (Salathe *et al.* 1993), which is phosphorylated by PKC and is involved in the regulation of CBF.

Thus we conclude that the regulation of CBF occurs via multiple interacting pathways dependent on phosphorylation. These interactions may have important implications for mucociliary clearance in both health and disease because many respiratory drugs, bacterial products and physical insults achieve their effects via these protein phosphorylation cascades.

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Author's email address

A. Mehta: a.mehta@dundee.ac.uk

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