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CALCIUM REGULATION OF CILIARY BEAT FREQUENCY IN HUMAN RESPIRATORY EPITHELIUM *IN VITRO*

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SUMMARY

1. The changes in ciliary beat frequency (CBF) of human nasal respiratory epithelial cells were measured *in vitro* with a photometric technique following exposure to either 4-bromo-calcium ionophore A23187 (4-Br-A23187) or trifluoperazine (TFP), an inhibitor of calmodulin-sensitive calcium-dependent protein kinases. Changes in intracellular free calcium concentrations in response to 4-Br-A23187 were studied using a fluorescent dye (Fura-2).

2. Addition of 10^{-5} M-4-Br-A23187 caused a time-dependent ($P < 0{\cdot}01$) rise in CBF. The increment in CBF was statistically significant 10 min after challenge ($+10\%$; $P < 0{\cdot}01$) and was sustained for at least 1 h, with maximal stimulation after 40 min ($+18\%$; $P < 0{\cdot}01$).

3. Exposure to 10^{-5} M-4-Br-A23187 caused an immediate increase in intracellular free calcium concentration, which preceded the rise in CBF.

4. TFP (10^{-4} M) caused a reduction of baseline CBF (-10%; $P < 0{\cdot}01$) and prevented the expected rise when the cells were subsequently exposed to 10^{-5} M-4-Br-A23187.

5. We conclude that: (1) calcium ionophore stimulates the CBF of human respiratory cells; (2) this effect is mediated through a calmodulin-sensitive system, since it is abolished in the presence of TFP; (3) the same pathway appears to control the basal CBF of these cells, since TFP also decreases CBF.

INTRODUCTION

Mucociliary clearance is one of the lung's non-specific host defence mechanisms, effected by the beating action of cilia propelling the overlying secretions, which carry both trapped inhaled material and locally produced biological debris toward the oropharynx (Wanner, 1977). Modifications of ciliary activity should lead to changes in rate of mucous transport, as predicted by theoretical models of mucociliary pumping (Ross & Corrsin, 1974). Consequently, the cellular mechanisms that regulate ciliary beat frequency (CBF) may also be considered as mechanisms regulating mucociliary clearance.

Neurohormones and neurotransmitters associated with the autonomic nervous

system have a significant influence on mucociliary function (Sleigh, Blake & Liron, 1988). There is very little information on the second messengers which might regulate ciliary activity in mammals, but is has been suggested that calcium and cyclic nucleotides might both be important in the control of CBF (Sanderson & Dirksen, 1989). Studies on unicellular organisms (Eckert, 1972; Machemer, 1974) and marine mussels (Satir, 1975) have indicated that intracellular calcium plays a pivotal role in the control of ciliary activity. In these simple organisms, intracellular calcium has a complex effect on the direction and frequency of ciliary beating. In mammalian ciliated epithelium, where the direction of ciliary beating is fixed (Sleigh, 1984), a rise in intracellular calcium increases the CBF (Girard & Kennedy, 1986; Sanderson & Dirksen, 1989) and blockers of calmodulin-dependent kinases induce a fall in the basal CBF (Girard & Kennedy, 1986). There appear to be no studies on the effects of changes in intracellular calcium concentration on human respiratory ciliary activity.

In this study we investigated: (1) the role of calcium in the regulation of CBF in human nasal respiratory epithelial cells using 4-bromo-calcium ionophore A23187 (4-Br-A23187) to raise intracellular calcium; (2) the relationship between calcium ionophore-induced changes in CBF and intracellular free calcium concentration; (3) the effect of an inhibitor of calmodulin-dependent kinases (trifluoperazine, TFP) (Levin & Weiss, 1976) on CBF.

METHODS

Sample collection

Ciliated nasal epithelium was obtained with a cytology brush, using a technique not requiring anaesthesia described by Rutland & Cole (1980). Two or three brushings were taken from the same nostril of each subject. Cellular material adhering to the brush was dislodged by brisk agitation in Eppendorf tubes containing 1 ml of tissue culture medium (Medium 199). All the experiments were performed within 4 h of collection. During this time the samples were kept in Medium 199 at 4 °C.

Subject selection

The material for the study was obtained from three groups of ten healthy volunteers. None of them had a history of upper respiratory tract infection for at least 1 month. The first group consisted of six men and four women, aged 23–50 years (mean 32·3 years). Samples from these subjects were used to test the effect of 4-Br-A23187 on CBF. The second group of subjects consisted of seven men and three women, aged 18–32 years (mean 25·8 years). Samples obtained from these subjects were used to measure the intracellular free calcium concentration at baseline and after exposure to 4-Br-A23187. The third group included five men and five women aged 18–42 years (mean 27·6 years). Cells from these subjects were used to test the effect of TFP on CBF. Informed consent was obtained from the subjects before collection.

Measurement of ciliary beat frequency

Ciliated cells were transferred in Medium 199 to a glass-walled perfusion chamber (Prior modified, UK) with an internal volume of 0·35 ml. The chamber was connected to a perfusion pump delivering 0·25 ml min^{-1}, which gave a 90% wash-out of the fluid in the chamber in 10 min. The wash-out time was calculated by filling the chamber with a coloured solution and measuring the rate of decline of absorbance during perfusion with distilled water. CBF was measured at room temperature (23·1 ± 1·3 °C) using a photometric technique (Dalhamn & Rylander, 1962; Di Benedetto, Gill, Lopez-Vidriero & Clarke, 1989). The sample preparation was placed on the stage of a phase-contrast microscope (Leitz Dialux 20, Germany), fitted with a photomultiplier connected to an amplifier (Leitz MPV Compact Microscope Photometer). Light directed from

below and passing through the specimen was interrupted by the sweeping action of the cilia. Variations in light intensity, corresponding to the beat frequency, were detected by the photomultiplier, transduced to electric impulses, and amplified. The electrical signal was fed through a low-pass filter into an ultraviolet oscillograph which provided a permanent record of ciliary activity. CBF values were obtained by counting the number of ciliary beats recorded in 10 s intervals and were expressed in Hz. In addition to the 'manual' counting, CBF values were determined by Fast Fourier Transform analysis of the signal (Kennedy & Duckett, 1981). The two methods provided similar CBF values, with differences within 0·2 Hz ($r = 0{\cdot}982$; $n = 40$). In order to eliminate the increase in CBF induced by perfusion (Di Benedetto, Lopez-Vidriero & Clarke, 1987) all measurements were taken in the absence of flow. In this preparation the CBF may be studied in real time when the cells are exposed to a number of different agents. The same group of cilia in each preparation was studied throughout the experiment, i.e. the relative position of the photometer's field diaphragm (approximately $1{\cdot}5 \times 5$ μm) and the selected epithelial border was kept constant. The longitudinal axis of the photometer's field was orientated perpendicularly to the cell border and CBF measurements were taken from clumps of two of more cells with free border devoid of debris. The choice of the ciliated area was randomized by using a eyepiece graticule and selecting the group of cilia which lay closest to the crosspiece of the graticule on the equatorial line. Following a 30 min period of equilibration on the microscope stage, the sample in the chamber was perfused for 10 min with fresh Medium 199 equilibrated with room air. Baseline CBF values were then obtained by taking the mean of measurements after 1, 15 and 30 min. The pH of all solutions tested was between 7·2 and 7·4, a range known not to affect CBF (Luk & Dulfano, 1983). The extracellular Ca^{2+} concentration was 1·8 mM, a value within the range known not to affect the basal CBF in rabbit tracheal explants (Girard & Kennedy, 1986). All the CBF measurements were taken by the same observer, who was unaware of the nature of the perfusates.

Effect of calcium ionophore on ciliary beat frequency

Following the baseline readings, the perfusate was replaced with either Medium 199 containing the vehicle or Medium 199 supplemented with 4-Br-A23187 (10^{-5} M). The calcium ionophore was dissolved in dimethyl sulphoxide (DMSO) and both solutions contained a final concentration of 1 % DMSO, a concentration known not to affect CBF (Di Benedetto, Manara-Shediac & Mehta, 1990*a*). CBF was measured after 1 min (following the 10 min period of perfusion) and then every 10 min for 1 h.

Effect of trifluoperazine on ciliary beat frequency

Following the baseline readings, the perfusate was replaced with either Medium 199 or Medium 199 supplemented with TFP (10^{-4} M). CBF measurements were taken 1 min after perfusion and then every 15 min for 1 h. After this period of observation, the perfusate was replaced with Medium 199 supplemented with 4-Br-A23187 (10^{-5} M) and the CBF was measured after 1 min (following the 10 min period of perfusion) and then every 10 min for 1 h.

Measurement of intracellular free calcium concentrations

Cells were loaded with the calcium indicator Fura-2 by incubation with a permeant form of the dye (1·0 μM-acetoxymethyl ester) in Medium 199 for 30 min at 37 °C in a shaking waterbath. The cells were subsequently centrifuged, resuspended, washed twice and then plated onto poly-*l*-lysine coated 32 mm square glass cover-slips. The cover-slips were mounted on the stage of an inverted microscope (Nikon Diaphot, Japan) and the cells were viewed at 400 × using a Nikon 40 × 1·3 NA phase-contrast oil immersion fluorescence objective. Beating ciliated epithelial cells were selected from other cell types by their characteristic appearance. After selection, each cell was positioned in the light path of a photomultiplier (Thorn EMI 9924B) and the path was narrowed to the area of the cell using an iris diaphragm to exclude stray or scattered light and to optimize the signal ratios. The cell was then illuminated alternately with 360 and 390 nm narrow band filtered (Ealing Electro-optics filters) ultraviolet light from a 150 W Xenon source fed via a fluid filled light guide (Micro Instruments Oxford) through the epifluorescence port of the microscope. Filters were changed by a solenoid operated filter changer every 250 ms. The ultraviolet light was reflected onto the cell by a 430 nm dichroic mirror and emitted light passed from the cell through a 470 nm barrier filter, then a 500 nm broad-band filter, before reaching the photomultiplier. The photomultiplier signals were fed into an IBM-AT compatible personal computer, after digitization by a CED 1401

Laboratory Interface (Cambridge Electronic Design). The same equipment also controlled the filter movement. The ratio of the emission signals at 360 and 390 nm was used to calculate the intracellular free calcium concentration, $[Ca^{2+}]_i$, from the equation:

$$[Ca^{2+}]_i = K_d(F_0/F_1)(R-R_{min})/(R_{max}-R),$$

where R_{max} was the ratio value at saturating calcium, R_{min} the ratio value at limitingly low calcium, (F_0/F_1) the ratio of fluorescence at 390 nm in low calcium to that in high calcium, and K_d the calcium dissociation constant for Fura-2 (Grynkiewicz, Poenie & Tsien, 1985). These values were obtained by the method of Almers & Neher (1985) from rat parotid acinar cells, using the whole-cell patch clamp method of dye loading and the free acid form of Fura-2 as the calcium indicator. In repeated calibrations with rat parotid acinar cells *versus* those made with cell-free solutions of known calcium concentrations, the 390 nm ratio values varied by less than 5%. Before analysis, each cell record was further calibrated by subtraction of the mean background autofluorescence recorded from cells not loaded with the dye. These were remeasured prior to each experiment, and found to be constant at 2·5% of the resting Fura-loaded cell fluorescence. All recordings were made from cells under continuous gravity-fed perfusion at 0·2–0·3 ml min^{-1} with either Medium 199 containing the vehicle or Medium 199 supplemented with 10^{-5} M-Br-A23187. We used the more efficient 4-bromo form of the calcium ionophore (Debono, Molloy, Dorman, Paschal, Babcock, Deber & Pfeiffer, 1981) to avoid the fluorescence interference artifacts associated with the non-halogenated form (Deber, Tom-Kun, Mack & Grinstein, 1985) and to optimize calcium uptake. The perfusion out-flow tip was positioned within 200 μm of the cell, as observed under the microscope, and complete solution changes in the region of the cell were achieved within 2 s.

Chemical reagents

Medium 199 and fura-2 were obtained from Flow Laboratories (Rickmansworth) and Molecular Probes Inc. (Eugene, OR, USA), respectively. DMSO, 4-Br-A23187, TFP, and poly-*l*-lysine were purchased from Sigma (Poole).

Statistical analysis

CBF and $[Ca^{2+}]_i$ values were expressed as mean ± S.E.M. and the response as the percentage change from the baseline. Differences in values were determined by a paired Wilcoxon's test and time–response relationships were determined by a Spearman correlation test (Siegel & Castellan, 1989). Significance was accepted when $P < 0·05$.

RESULTS

Effect of calcium ionophore on ciliary beat frequency

The baseline CBF (7·1 ± 0·3 Hz) was unchanged after perfusion with control solution (Fig. 1). In these experiments the mean intra-cell coefficient of variation of the frequency, defined as the ratio of the standard deviation to the mean value as a percentage, was ±6·3%. Perfusion with 10^{-5} M-4-Br-A23187 caused a time-dependent ($P < 0·01$) rise in CBF from a baseline of 6·8 ± 0·3 Hz (Fig. 1). The rise in CBF was statistically significant 10 min after perfusion (+10%; $P < 0·01$) and continued to increase until 40 min (+18%; $P < 0·01$), with a plateau thereafter. All CBF values after 10 min were significantly higher than baseline ($P < 0·01$). At the end of the observation period (1 h after perfusion) the CBF was still significantly elevated in comparison with the baseline values (+16%; $P < 0·01$).

Effect of calcium ionophore on intracellular free calcium

Exposure of the cells to control solution did not significantly affect the basal $[Ca^{2+}]_i$ (86 ± 7 nM; $n = 25$) (Fig. 2). Exposure to 10^{-5} M-4-Br-A23187 caused a statistically significant increase of $[Ca^{2+}]_i$ (from a baseline of 64 ± 14 nM; $n = 25$)

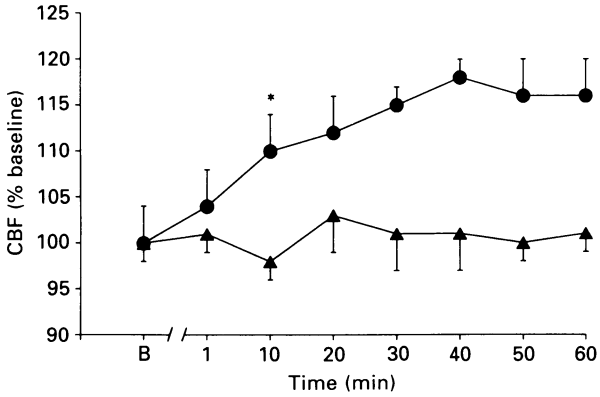

Fig. 1. The effects of control solution (▲) and 10^{-5} M-4-Br-A23187 (●) on baseline (B) CBF of human nasal epithelium. Each data point represents the mean ± S.E.M. ($n = 10$). On the y-axis the 100% value represents mean baseline CBF of 7·1 and 6·8 Hz for the control and 4-Br-A23187 experiments, respectively. The break in the x-axis represents the perfusion period. CBF did not change after perfusion with control solution. Perfusion with 4-Br-A23187 caused a time-dependent ($P < 0{\cdot}01$) increase in CBF, that reached statistical significance after 10 min (+10%; $P < 0{\cdot}01$). Maximal stimulatory effect was reached after 40 min (+18%; $P < 0{\cdot}01$), with a plateau thereafter. *$P < 0{\cdot}01$.

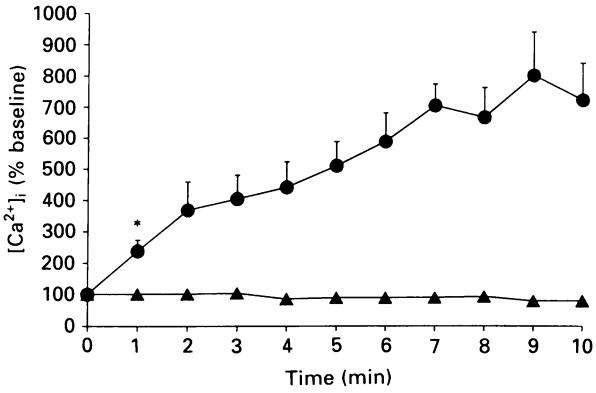

Fig. 2. The effect of control solution (▲) and 10^{-5} M-4-Br-A23187 (●) on the $[Ca^{2+}]_i$ of human nasal cells. Each data point represents the mean ± S.E.M. for cells randomly selected from 10 subjects ($n = 25$). Exposure to control solution did not change $[Ca^{2+}]_i$. Exposure to the calcium ionophore caused an immediate increase in $[Ca^{2+}]_i$, which reached maximal values after 9–10 min. *$P < 0{\cdot}01$.

within 1 min (+139%; $P < 0{\cdot}01$) (Fig. 2). The $[Ca^{2+}]_i$ continued to rise up to 10 min (+623%; $P < 0{\cdot}01$), after which there was a plateau which was sustained for at least 10 min.

Effect of trifluoperazine on ciliary beat frequency

Perfusion with 10^{-4} M-TFP caused a reduction in CBF with respect to a baseline of 7·2 ± 0·2 Hz (Fig. 3). The decrease in CBF was statistically significant 30 min after perfusion (−7%; $P < 0{\cdot}05$). Minimal CBF values were reached after 45 min (−10%;

$P < 0{\cdot}01$), with a plateau thereafter. At the end of the observation period (1 h after perfusion) the CBF was still reduced relative to the baseline (-9%; $P < 0{\cdot}01$). Subsequent perfusion with 10^{-5} M-4-Br-A23187 did not change CBF (Fig. 3) and the CBF remained below baseline values (-10%; $P < 0{\cdot}01$) for the whole period of

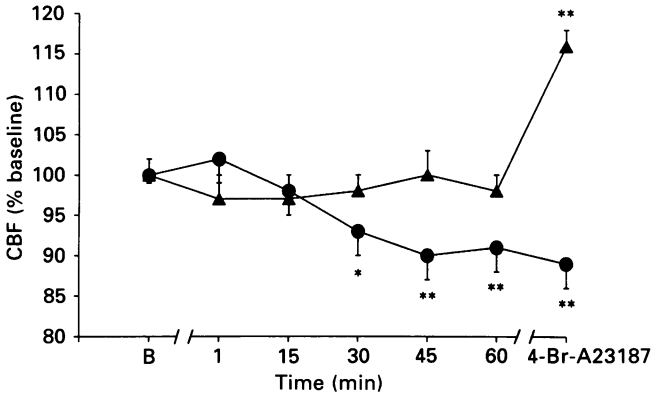

Fig. 3. The effect of control solution (▲) and 10^{-4} M-TFP (●) on baseline (B) CBF of human nasal epithelium. Each data point represents the mean ± S.E.M. ($n = 10$). On the y-axis the 100% value represents mean baseline CBF of 7·0 and 7·2 Hz for the control and TFP experiments, respectively. The breaks in the x-axis represent the perfusion periods. Perfusion with control solution did not change CBF. Subsequent perfusion with 10^{-5} M-4-Br-A23187 increased the CBF, with a time course similar to that illustrated in Fig. 1. For clarity, the calcium ionophore data point (4-Br-A23187) illustrated in the above figure represents the CBF value 40 min after challenge, when maximal stimulatory effect was observed. Perfusion with TFP caused a reduction in CBF, that was statistically significant after 30 min (-7%; $P < 0{\cdot}05$). Minimal values were recorded after 45 min (-10%; $P < 0{\cdot}01$), with a plateau thereafter. Subsequent perfusion with 10^{-5} M-4-Br-A23187 failed to increase CBF. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

observation (1 h). At the end of each experiment, the viability of the cells was assessed by increasing the temperature in the chamber to 37 °C using a warm stage. The temperature increment caused an immediate (within 1 min) rise of CBF ($+55\%$; $P < 0{\cdot}01$).

Incubation for 1 h with control solution did not affect the expected rise following perfusion with 10^{-5} M-4-Br-A23187 (Fig. 3).

DISCUSSION

Calcium regulation of ciliary activity

Calcium is an important regulator of cell function (Rasmussen, 1986*a*, *b*) and is involved in the control of both flagellar (Tash & Means, 1983) and ciliary (Verdugo, Raess & Villalon, 1983; Kakuta, Kanno, Sasaki & Takishima, 1985; Girard & Kennedy, 1986; Sanderson & Dirksen, 1989) activity, but its effects vary with the type of cell or tissue examined. In *Paramecium*, Ca^{2+} regulates the direction of the effective stroke and elevated intracellular Ca^{2+} levels stimulate CBF (Eckert, 1972; Machemer, 1974). In contrast, the lateral cell cilia of *Mytilus* gill are arrested by an

increase in intracellular calcium (Satir, 1975). Several studies indicate that Ca^{2+} control of ciliary activity in ciliated epithelia of vertebrates may in most cases differ from that of protozoa and mussel gills (Girard & Kennedy, 1986).

Effect of calcium ionophore on ciliary beat frequency

In our study, addition of 10^{-5} M-4-Br-A23187 led to an increase in CBF and this response had some similarities with that seen in rabbit tracheal cells (Girard & Kennedy, 1986; Sanderson & Dirksen, 1989). Girard & Kennedy (1986) challenged rabbit tracheal explants with 10^{-5} M-A23187 and observed an immediate increase in CBF with a gradual decrease after 20–30 min, although CBF remained above baseline levels for 1 h. In our experiments, the rise in CBF had an onset between 1–10 min after challenge and reached its maximum after 40 min. The time course of the ionophore-dependent increase of CBF was different from that observed in our previous studies on the stimulation of CBF through cyclic AMP-dependent mechanisms (Di Benedetto, Manara-Shediac & Mehta, 1990*b*), where 10^{-3} M-dibutyryl cyclic AMP induced an immediate (within 1 min) maximal increase in CBF. It is also interesting to note that, although the magnitude of the increase caused by calcium ionophore was smaller (+18%) in comparison to that reported by Girard & Kennedy (1986) in rabbit tracheal cells (+30%), it was similar to that observed in our previous study with dibutyryl cyclic AMP (+15%) (Di Benedetto *et al.* 1990*b*). On a percentage basis, these changes of CBF in human cells appear to be small. However, this need not necessarily be taken to imply that such increments might be physiologically insignificant, especially when additional frequency-dependent factors influencing mucociliary transport (ciliary coordination, length of the metachronal wave, effective/recovery stroke time-ratio) are taken into account (Sleigh *et al.* 1988). In a recent study (Seybold, Mariassy, Stroh, Kim, Gazeroglu & Wanner, 1990) it has been shown that an increase of 16% in CBF induced by 10^{-3} M-acetylcholine resulted in a simultaneous 56% acceleration of surface liquid velocity in an *in vitro* preparation of sheep trachea. Furthermore, the authors demonstrated that the magnitude of the changes in one component of mucociliary interaction cannot predict those of other components. It is, therefore, not possible at present to evaluate the physiological *in vivo* significance of our observed changes.

Effect of calcium ionophore on intracellular free calcium

The uptake of $^{45}Ca^{2+}$ is increased by A23187 in several tissues (Reed & Lardy, 1972; Prince, Rasmussen & Berridge, 1973) and, at low concentrations, A23187 causes a release of Ca^{2+} from an intracellular Ca^{2+} pool (Babcock, First & Lardy, 1976; Stolze & Schulz, 1980). We calculated $[Ca^{2+}]_i$ using values obtained by the method of Almers & Neher (1985) from rat parotid acinar cells, using the whole-cell patch clamp method of dye loading and the free acid form of Fura-2 as the calcium indicator. Calibration using this method has so far proved impossible with ciliated airway epithelial cells, due to the difficulty in achieving gigaohm seals on these cells with microelectrodes of sufficiently low resistance to allow formation of the whole-cell recording configuration and adequate intracellular dialysis. The relative effects of recording from spherical cells should be similar with these two cell types, as they

are of similar dimensions, but specific differences in intracellular influences on Fura-2 cannot be ruled out. For this reason, although the basal $[Ca^{2+}]_i$ measured in our study is of the same order of magnitude as that reported by others (Murphy, Cheng, Yankaskas, Stutts & Boucher, 1988), absolute values obtained could only be taken as best available estimates. However, the relative changes in calcium concentration (expressed as a percentage of baseline) would be unaffected by the lack of absolute calibration standards.

Exposure of human nasal cells to 10^{-5} M-4-Br-A23187 was followed by an immediate increase in $[Ca^{2+}]_i$, which reached a plateau after 10 min. Exposure of the cells to the calcium ionophore also increased CBF, but the time course of the rise in $[Ca^{2+}]_i$ and the increment in CBF differed, in that the rise in $[Ca^{2+}]_i$ preceded the change in CBF. Although the experiments on CBF and $[Ca^{2+}]_i$ were not performed simultaneously, our results suggest that at the end of the 10 min period of perfusion, when the concentration of 4-Br-A23187 in the chamber was 10^{-5} M, the $[Ca^{2+}]_i$ would already have been increased above baseline and yet there was no significant increase in CBF. There are several possible explanations for this, including a threshold effect for the increase in $[Ca^{2+}]_i$ or a delay in the full activation of calmodulin-dependent kinases following the initial rise in $[Ca^{2+}]_i$, mediated by autophosphorylation of the kinases (Colbran, Schworer, Hashimoto, Fong, Rich, Smith & Soderling, 1989). Our results offer preliminary evidence that the latter mechanism is more likely, since maximal Ca^{2+} levels were detected after 10 min of exposure to the calcium ionophore, whereas maximal CBF stimulation was observed much later (40 min).

Effect of trifluoperazine on ciliary beat frequency

TFP is known to antagonize the action of calmodulin-sensitive Ca^{2+}-dependent enzymes (Levin & Weiss, 1976). We used TFP to probe for possible involvement of calmodulin-dependent pathways in the motility of cilia in the basal state. We observed an inhibition of CBF after exposure to 10^{-4} M-TFP, which is in agreement with the findings of Girard & Kennedy (1986) in rabbit tracheal explants. The magnitude of the reduction of CBF, however, was much smaller in our study, with a maximal decrease of -10% in comparison to -50% reported by the above workers. The characteristics of the TFP-dependent inhibition of the basal CBF (with a plateau 10% below baseline) together with the ability of the ciliated cells to respond to the rise in temperature, a physical stimulus known to increase the activity of ciliated cells (Kennedy & Duckett, 1981), suggest that TFP did not cause 'toxic' damage to the cells. The importance of calmodulin-sensitive Ca^{2+}-dependent enzymes in the control of CBF in mammalian respiratory epithelium is also supported by the studies of Verdugo and colleagues on rabbits cells (1983), where they observed an increase in the effect of Ca^{2+} on the CBF of demembranated tracheal cilia by adding exogenous calmodulin. The complete abolition of the cilio-stimulatory effect of 4-Br-A23187 in presence of TFP observed in our study suggests that the ionophore might activate a calmodulin-sensitive system.

The effects of TFP are not confined to the calmodulin-dependent kinases. TFP antagonizes the action of phospholipid-sensitive Ca^{2+}-dependent protein kinase (PKC) (Schatzman, Wise & Kuo, 1981) in addition to calmodulin-dependent enzymes. Therefore, it remained possible that the effect of TFP on CBF might be due to PKC inhibition. This, however, is unlikely, since our previous studies suggest that

PKC plays no major role in the control of basal CBF in humans (Di Benedetto *et al.* 1990*a*). In these studies, using the same chamber preparation, we showed that 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H-7) (10^{-5}–10^{-3} M), an inhibitor of cyclic nucleotide-dependent kinases and of PKC (Hidaka, Inagaki, Kawamoto & Sasaki, 1984), did not affect the basal CBF, an observation reported also by others in rabbit tracheal explants (Kobayashi, Tamaoki, Sakai, Chiyotani & Takizawa, 1988). H-7 (10^{-4} and 10^{-3} M), however, was able to block a cyclic AMP-dependent increase in CBF. We also demonstrated that stimulation of PKC with phorbol myristic acid (10^{-5} M) did not change CBF (Di Benedetto *et al.* 1990*a*). The combination of the results on the effects of H-7, phorbol myristic acid and TFP suggest that the inhibition of the basal CBF in the presence of TFP was mediated through a calmodulin-kinase system.

In conclusion we have shown that calcium ionophore stimulates the CBF of human respiratory epithelial cells, probably through a calmodulin-sensitive system, since this response was abolished in the presence of TFP. Moreover, since TFP reduced the basal CBF, whereas an inhibitor of cyclic nucleotide-dependent kinases and of PKC-dependent enzymes had no effect of CBF (Di Benedetto *et al.* 1990*a*), we suggest that the calmodulin Ca^{2+}-dependent pathway is responsible for the control of the basal ciliary activity of these cells.

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