HUMAN RESPIRATORY EPITHELium: CONTROL OF CILIARY
ACTIVITY AND TECHNIQUES OF INVESTIGATION

by

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ABSTRACT

In man, most of the upper airways and the tracheo-bronchial tree down to the non alveolar walls of the respiratory bronchioles are covered by ciliated epithelium. Mucociliary clearance is the most important clearing mechanism of the respiratory tract and is the result of beating cilia propelling the overlying secretions, carrying both trapped inhaled material and locally produced biological debris, toward the oropharynx. The rate of mucus transport is determined by the power produced by each cilium and the number of cilia in contact with the mucus. Both of these parameters are a function of ciliary beat frequency (CBF). This thesis deals with a technique for measuring CBF in vitro and how CBF is regulated in human respiratory cells. We measured CBF with a photometric technique and used a perfusional apparatus to observe changes in CBF in response to modifications of the extra- and intra-cellular microenvironments. We investigated the variability of CBF in human respiratory cells and assessed the reliability of a technique for storing ciliated cells at sub-zero temperatures, without affecting the basal CBF and the ciliary response to pharmacological stimulation. Our apparatus was tested in a study on the effect of Amiloride on ciliary activity, where we demonstrated a cilio-stimulatory action of the drug. The last two Chapters describe experiments on the role of cyclic AMP and calcium in regulating CBF in human respiratory epithelium. We assessed dose-response and time-response curves and used inhibitors of
cyclic nucleotide- and calcium-dependent kinases to further clarify the intra-cellular pathways. The results indicate that in man, as in other species, both cyclic AMP and calcium regulate CBF.
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<th>Description</th>
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<tr>
<td>AM</td>
<td>Amiloride</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine triphosphatase</td>
</tr>
<tr>
<td>4-Br-A23187</td>
<td>4-bromo-calcium ionophore A23187</td>
</tr>
<tr>
<td>[Ca\textsuperscript{2+}i]</td>
<td>Free intracellular calcium concentration</td>
</tr>
<tr>
<td>CALLS</td>
<td>Correlation analysis laser light-scattering</td>
</tr>
<tr>
<td>CBF</td>
<td>Ciliary beat frequency</td>
</tr>
<tr>
<td>CO\textsubscript{2}</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CoV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>Cyclic AMP</td>
<td>Adenosine 3':5'-cyclic monophosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>g</td>
<td>Gravitational constant</td>
</tr>
<tr>
<td>Gy</td>
<td>Guery (100 rads)</td>
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<tr>
<td>H-7</td>
<td>1-(5-isoquinolinylsulphonyl)-2-methylpiperazine</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz (cycle/s)</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>K\textsubscript{d}</td>
<td>Concentration necessary to produce a half-maximal response</td>
</tr>
<tr>
<td>LT</td>
<td>Leukotriene</td>
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<tr>
<td>MBP</td>
<td>Major basic protein</td>
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<td>MCC</td>
<td>Mucociliary clearance</td>
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<tr>
<td>Symbol</td>
<td>Definition</td>
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<tr>
<td>--------</td>
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</tr>
<tr>
<td>n</td>
<td>Number of observations</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetylcysteine</td>
</tr>
<tr>
<td>NS</td>
<td>Not significant</td>
</tr>
<tr>
<td>P</td>
<td>Probability</td>
</tr>
<tr>
<td>PD</td>
<td>Potential difference</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristic acid</td>
</tr>
<tr>
<td>r</td>
<td>Correlation coefficient</td>
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<tr>
<td>SA</td>
<td>Spectrum analyzer</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SO₂</td>
<td>Sulphur dioxide</td>
</tr>
<tr>
<td>SRS-A</td>
<td>Slow-reacting substances of anaphylaxis</td>
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<tr>
<td>TFP</td>
<td>Trifluoperazine</td>
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* All units or abbreviations not included above are SI units
1. GENERAL INTRODUCTION

1.1. Introduction

Cilia were first seen in 1676 by the Dutch naturalist and microscope maker Antony van Leeuwenhoek, who sent to the Royal Society of London a letter describing his discovery of protozoa and their cilia and flagella (Haimo & Rosenbaum, 1981). In a review of 1835, Sharpey gave detailed descriptions of the activity of cilia in a wide variety of animals and reported the discovery of ciliary motion in the reproductive and respiratory systems of mammals in the previous year by Purkinje and Valentin (1834).

Apart from nematodes and some arthropods, cilia are ubiquitous throughout the plant and animal kingdoms. In many invertebrate animals, cilia perform a variety of functions including locomotion, food collection and transport of excretory products (Sleigh, 1962). Mammals employ cilia extensively for moving fluids over epithelial surfaces in respiratory and reproductive systems.

Cilia are cellular projections with intrinsic motility; they contain cytoplasmic fibrils whose active motion causes changes in the shape of the cilium, which results in propulsion of the fluid that overlies the cell (Sleigh, 1977).

In man, cilia are present in the upper (nasal passages, sinuses, middle ear and Eustachian tube) and lower (trachea to respiratory bronchioles) respiratory tracts. In the genital tract they are present in
the efferent ductules, the endometrium of the uterine cervix and they line the Fallopian tubes. Motile cilia also line the ependyma of the brain and spinal cord, although their function in these sites is not understood. Spermatozoa are modified cilia and retinal rods, vestibular hair cells and olfactory cells are derived from similar prodromal structures.

The introduction of electron microscopy dramatically improved the knowledge of ciliary structure and function (Sleigh et al., 1988): it was used to demonstrate the 9 plus 2 arrangement of internal fibrils in sections of cilia (Fawcett & Porter, 1954), to discover accessory structures within the axoneme (Afzelius, 1959), to support a sliding-fibril hypothesis of ciliary bending (Satir, 1965), and, combined with improved biochemical techniques, to show the localization of adenosine triphosphatase (ATPase) activity in dynein arms (Gibbons, 1965).

1.2. Mucociliary clearance

Mucociliary clearance (MCC) is the most important clearing mechanism of the respiratory tract and is the result of beating cilia propelling the overlying secretions, carrying both trapped inhaled material and locally produced biological debris, toward the oropharynx (Pavia, 1984) (Fig. 1.1.). If the amount of mucus is not excessive and the mucociliary escalator is functioning normally, then the clearance of mucus from the lungs and upper respiratory tract is accomplished by unconscious swallowing.

The nose is equipped morphologically and functionally to modify the
Fig. 1.1. Distribution of ciliated epithelium and direction of mucociliary transport (from Mygind, 1986).
inimical aspects of our ambient air and a major factor in this physiological function is MCC (Proctor, 1983). Ciliated cells appear in large numbers in the nasal epithelial surface, extending from somewhat posterior to the anterior ends of the middle and inferior turbinates to the nasopharynx. Even in the nasopharynx a few ciliated cells are found, especially over the adenoid tissue.

The human lung has several mechanisms whereby it can rid itself of inhaled deposited material and remain relatively clean and sterile even if exposed to polluted atmosphere. The three main clearance mechanisms operating in the lungs are a) mucociliary clearance, b) cough and c) alveolar clearance (Pavia et al., 1980).

On Weibel's model of the human lung (Weibel, 1963) (Fig. 1.2.) ciliated cells are present from the level of the trachea (generation 0) to the terminal bronchioles (16th generation). The total area covered by the ciliated respiratory epithelium is about 0.55 m² and the total number of ciliated cells per person has been estimated as about $3 \times 10^{12}$ (Afzelius, 1979). The proportion of ciliated cells in the surface epithelium varies with the size of the airways, but whether the variation is towards an increase or decrease is not clear. Jeffery and Reid (1975) found that in the rat airway there is a gradual increase from 17% in the upper trachea, to 35% in the extrapulmonary bronchi, 53% in the intrapulmonary and 65% in the bronchioli. In contrast, the studies of Serafini and Michaelson (1977) in human and canine airways have shown that the percentage of ciliated cells decreases from 53% in the trachea
Fig. 1.2. Stylized version of Weibel's model of the human lung (from Pavia et al., 1980).
to 45% in the first airway generation, 23% in the third and 15% in the fifth generation. The discrepancy between these findings could be due to a species difference and methodology (Lopez-Vidriero, 1984). Each ciliated cell has a diameter of 5µm or so at its apex and carries some 200 cilia at a density of 6 to 8 µm², interspersed with short microvilli (Rhodin, 1966; Jafek, 1983). The length of the cilia decreases from 6 µm in the trachea to 3.6 µm in the 7th generation bronchi (Serafini & Michaelson, 1977) and possibly even more so as one gets nearer to the periphery of the lung.

The observations of Lucas and Douglas (1934) on monkey ciliated epithelium suggested that cilia beat in a watery (sol) layer propelling forwards at their tips a viscid (gel) layer which may be continuous or consist of discrete islands. This finding was confirmed by studies on thick sections of rabbit tracheal epithelium with scanning electron microscopy (Sanderson & Sleigh, 1981).

Cilia propel fluids because the cyclical movements that they perform are asymmetric (Sleigh et al., 1988). The cilia beat with an active and a recovery phase (Satir, 1968). A quarter of each cycle is taken up by the cilia beating 'forward' pushing secretions towards the mouth and three quarters in bending 'backwards' ready for the next stroke. The rapid 'effective' stroke occurs mainly in one plane with the cilium erect above the cell surface. At the end of this stroke the cilium must return to its starting point to begin another effective stroke without engaging the mucus, which would cancel the propulsive effect of the previous effective
stroke. To satisfy this requirement the cilium returns to its starting point in a different plane from the effective stroke, sweeping at right angles across the cell surface within the periciliary fluid. Mucus-propelling cilia normally rest between beats, and scanning electron micrographs of rabbit tracheal epithelium show that this rest takes place after the effective stroke has been completed, so that the tip of the resting cilium points in the direction of the mucus flow (Sleigh, 1981). The cilia do not beat simultaneously, but in a coordinated fashion, one after the other, so producing metachronal waves travelling in the opposite direction to the effective stroke. Active cilia occur in coordinated groups, the coordination arising because some visco-mechanical coupling occurs between moving cilia that are fairly close together (Sleigh, 1976). Cilia carry lung secretions against gravity and can transport weights up to 10g cm\(^2\) without affecting their performance (Maxwell, 1905).

MCC rate varies from 10 to 24 mm/min in the trachea and from 4.5 to 7 mm/min in the nose (Proctor & Lundqvist, 1973; Wanner, 1977; Wilkey et al., 1980).

1.3. Mucus

Tracheo-bronchial mucus is a mixture of secretions from the surface epithelium, submucosal glands and tissue fluid transudate (Lopez-Vidriero, 1984). The volume of mucus produced daily by a normal human tracheo-bronchial tree is not known with certainty. It has been estimated that the normal volume of tracheo-bronchial secretions ranges from 10
to 100 ml/day (Kaliner et al., 1986).

Airway mucus consists of water, dialysable material and macromolecular constituents. Water is the main constituent by weight and in normal secretions it accounts for 95% of the total weight. Dialysable material -salts and small molecules- represents only 1%. Mucus glycoprotein (2-3%), proteins (0.1-0.5%) and lipids (0.3-0.5%) are the major macromolecular constituents (Lopez-Vidriero, 1984). The viscosity and elasticity characteristic of tracheo-bronchial secretions are due primarily to the presence of mucus glycoprotein. The mucus glycoprotein has a protein core with multiple oligosaccharide side chains cross-linked by disulphide and hydrogen bonds (Roberts, 1978). Hydrolysis of the side chains gives N-acetylglucosamine, N-acetylgalactosamine, galactose, fucose and sialic acid. Other macromolecules synthesized locally include lysozyme, lactoferrin and immunoglobulines (Ig), mainly IgA. Transudated proteins identified in respiratory secretions include albumin, alpha-1 antitrypsin, alpha-2 macroglobulin, haptoglobin and transferrin, as well as IgM, IgG and IgA (Havez & Roussel, 1976).

Mucus has the physical properties of both a solid and a liquid: with slight stress it will deform and then reform when the stress is removed like an elastic solid, but with greater applied stress it flows like a viscous liquid. It is non-Newtonian and probably thixotropic, so that its apparent viscosity becomes less as the applied stress increases, causing it to flow more readily.

It is unclear what constitutes the ideal mucus in terms of
transportability. The ideal rheological properties of mucus for efficient transport are high elasticity and low viscosity (Wilson, 1988); these may change during disease, for example becoming less elastic during viral infection (Sakakura, 1983) or more viscous during bacterial infection (Burgi, 1973; Wanner, 1977). High viscosity mucus appears to have little adverse effect on transport in the frog palate preparation, but elasticity correlates well with speed of transport (Dulfano & Adler, 1975). These results have been confirmed by theoretical models of mucociliary pumping (Ross & Corrsin, 1974). Mucus shows cohesion as well as elasticity, and a feature that appears to represent a combination of these two characteristics is the spinability or thread-forming properties of mucus (Puchelle et al., 1983). The surface properties, such as stickiness (Medici et al., 1973) and 'peelability' of mucus from the cilia, can also influence the flow of secretions (Clarke & Pavia, 1980).

1.4. Ciliary ultrastructure (Sleigh, 1977)

Cilia are cellular projections, containing fibrils surrounded by cytoplasm and enclosed by the membrane of the cell. A cilium consists of three main structural regions: the ciliary shaft, the basal body and the ciliary roots.

The ciliary shaft is composed of longitudinal fibrils - the axoneme - which has an highly characteristic structure of 9 outer and 2 central microtubules bound together by links into a regular and functional unit (Fig. 1.3.). The outer microtubules consist of two microtubules, one
Fig. 1.3. The structure of a cilium in transverse section: outer microtubules A and B, nexin links (n), radial spokes (r), outer (o) and inner (i) dynein arms (modified from Sleigh, 1977).
complete (microtubule A) and one incomplete (microtubule B) (Gibbons & Grimstone, 1960). These microtubules are attached laterally to each other to make a doublet. Microtubules A and B are in turn composed of 13 protofilaments and 4 or 5 protofilaments are shared by both microtubules. The microtubules are composed of a protein called tubulin (Mohri, 1968). Two types of tubulin have been identified in both the A and B outer fibrils. The two central microtubules are also made up of protofilaments containing tubulin, which differs in its amino acid composition from those of the outer fibrils. The tubulins are structural proteins with no enzymatic function, although they have a site to which the dynein molecule can firmly bind and another site at which dynein interacts with tubulin in an ATPase dependent fashion (Afzelius, 1983).

Functional structures exist between outer microtubules and between outer and central tubules. Dynein arms (Afzelius, 1959; Gibbons & Rowe, 1965) connect the A microtubule to the B microtubule of the adjacent doublet; pairs of arms are borne at intervals of 17 nm along the A microtubule. The outer dynein arm is longer than the inner arm and has an extra segment. Biochemical and electron microscopic studies have shown that the dynein arms consist of ATPase or dynein protein (Gibbons, 1965). It has been estimated that each dynein arm is a multisubunit aggregate containing from 6 to 19 individual polypeptides (Warner, 1981). At least some of these polypeptides must have the ability to undergo conformational changes, which will then cause the dynein arm to push the neighbouring doublet a quantum step forward and this gives
rise to the sliding of the microtubular doublets.

The nine microtubular doublets have to be held together by elastic links in order that the cilia bend rather than slide apart. Some links have indeed been found and can be discerned, although with some difficulty, in most cross-sections of cilia examined at high magnification. The corresponding protein has been isolated and called nexin (Gibbons, 1965). The links have been called either nexin links or inter-doublet links. The nexin molecule represents 2% of the mass of the axoneme.

In addition, from the A microtubule an accessory structure -radial link or radial spoke- projects centrally toward the central tubules (Afzelius, 1959). The radial spoke terminates in a dilated breach. The single central microtubules also bear lateral projections in the form of curved rods, that are attached along the length of each microtubule in such a fashion that they appear to form an incomplete central sheath. The heads of the radial links appear to make connection with these projections from the central fibrils.

This characteristic axonemial structure exists all along the cilium except at the tip and base. At the tip of the cilium the microtubule B first terminates and the peripheral outer doublets become single microtubules, then the microtubules A disappear and only the central microtubules remain. A crown of 3 to 7 short 'claws' 25 to 35 nm long has been identified at the tips of the cilia (Jeffery & Reid, 1977; Foliguet & Puchelle, 1986). These projections penetrate into the mucus during the effective stroke.
At the ciliary base the axoneme continues into the basal body in a modified form. The central fibrils terminate near the level of the cell surface. The peripheral fibrils extend throughout the length of the basal body, but below the level of the cell surface each doublet becomes a triplet by the addition of another incomplete (C) microtubule attached alongside the B microtubule. The structure of the basal body is identical with the structure of the centriole that is concerned with the organization of the mitotic spindle at nuclear division. In many types of ciliated cells products of the replication of "nuclear" centrioles migrate to the cell surface and act as the organizing centres for ciliary axonemes. A fundamental question has been raised as to whether the basal bodies contain ribonucleic acid; this has not been satisfactorily answered (Hartman & Gurney, 1974). A specialized region of the ciliary membrane -ciliary necklace- has been identified in somatic cilia in the transition region where the ciliary membrane joins the plasma membrane and where extensions from the outer doublets to the ciliary membrane are present. Comparative studies suggest that the ciliary necklace may be involved in the control of localized membrane permeability and may play a role in the timing of ciliary beat (Gilula & Satir, 1972).

The main anchorage of the axoneme is provided by "root" fibres attached to the basal body. The fibres are proteinaceous and show a marked cross-striation. The basal bodies frequently carry a lateral "basal foot", that projects as a cone from near the midregion of the basal body. Microtubules are attached to the tip of the basal foot and extend away
into the superficial cytoplasm of the cell. The basal foot of the cilium is found at that side of the basal body toward which the cilium bends in its effective stroke (Gibbons, 1961), and since all of the basal feet on a single cell are normally aligned in approximately the same direction (Holley & Afzelius, 1986), the effective strokes of all cilia on the cell have a common orientation. However, neither the orientation of the basal feet (Holley & Afzelius, 1986) nor the orientation of the beat (Sanderson & Sleigh, 1981) are precisely identical on adjacent cells. Larger striated roots generally extend from the inner end of the basal body toward the cell nucleus.

1.5. Mechanism of ciliary bending

The elements responsible for the bending movements of a cilium are present within the ciliary axoneme. Cilia that have been removed from their parent cell above the level of the basal body, and subsequently demembranated, are still able to beat when provided with adenosine 5'-triphosphate (ATP) and an appropriate ionic environment. Current hypotheses assume that bending is caused by active sliding of the peripheral doublets relative to one another. The first evidence for the sliding microtubule model was obtained by Satir (1965).

The sliding microtubule hypothesis assumes that the length of the microtubules remains constant and that when the microtubule doublets are caused to slide relative to one another the cilium bends because of cross-linking connections between the doublets.
The most likely explanation for the ATP-induced active sliding of the microtubules appears to be that the dynein arms generate a shearing force between adjacent doublets (Afzelius, 1983). The dynein molecules anchored to the A microtubule of one doublet are capable of cyclically changing shape as they bind and hydrolyse ATP, in a reaction sequence that is coordinated with the formation and breakage of linkages with binding sites on the B microtubule of the adjacent doublet. Changes in shape or angular orientation of the dynein molecules in the period while they are attached distally to the B microtubule can generate the required shearing force: one doublet moves along an adjacent one as its dynein arms make contact, swing and release.

The beat direction of cilia is perpendicular to the axis through the central tubules (Satir, 1965). Ciliary orientation can be estimated using electron microscope micrographs by measuring the angle between the plane defined by the central tubules and a reference line (Rautiainen et al., 1986; Rautiainen, 1988).

The activity of the dynein molecules requires ATP as a specific energy source. The ATP supply available within the organelle is limited (Brokaw, 1965; Goldstein et al., 1970) and continued motility is dependent upon ATP diffusing into the cilium from the cell body. The rate of diffusion of ATP within a cilium is adequate to maintain the ATP concentration above the level required for dynein activity (Brokaw, 1966; Nevo & Rikmenspoel, 1970). The presence of adenylate kinase and arginine kinase or creatine kinase could provide a means of mobilizing
a short-term reserve of high-energy phosphate and overcome irregularities in the energy supply or demand.

The radial links and nexin bridges do not take part directly in the production of forces of active sliding, but the radial links play an important role by limiting and regulating the sliding activities of the microtubules. They might also be responsible for the propagation of bending along the axoneme.

1.6. Coordination of cilia

An active ciliated epithelium is recognizable by the progression of waves of movement across the ciliated surface (Sleigh, 1974; Aiello & Sleigh, 1977; Sanderson & Sleigh, 1981; Marino & Aiello, 1982). The beat of each cilium is metachronally coupled to that of neighbouring cilia (Sanderson & Sleigh, 1981). The formation and propagation of metachronal waves is due to hydrodynamic forces between the moving cilia. If we consider only two adjacent cilia, we find that when one moves it influences its neighbour through viscous drag communicated through the intervening fluid (Machemer, 1974a). When one cilium starts to move from the rest position it pushes or pulls its neighbour in the same direction and causes it to start its recovery stroke with only a small phase difference between them. The same principles can be extended to explain coordination of all of the many cilia of a cell (Sleigh, 1983).

When observing a ciliated epithelium that is transporting mucus it is easy to determine the direction of the effective stroke of the cilia from
the direction of mucus transport, but the more visible phase of the ciliary beat is the recovery stroke, which moves in the opposite direction to the flow of mucus. The metachronism of cilia that transport mucus is irregular, and it is possible that the degree of coupling between cilia and the character of the metachronism could vary with the frequency of beating (Sleigh, 1984).

1.7. Factors affecting ciliary activity

Rather little of the knowledge available about the control of ciliary activity is derived from studies on mucus-propelling cilia, because they are more difficult to study than the water-propelled cilia found in many groups of invertebrate animals. However, much of this information is not applicable to mucus-propelling cilia, because their movement and coordination are different, adapted to the function of mucus transport (Sleigh, 1981).

1.7.1. Physical and chemical factors

Temperature. Numerous studies have been conducted on temperature effects on ciliary activity. Probably the most complete report in this respect has been by Mercke and colleagues (1974a) on rabbit trachea, where they recorded mucociliary wave movements indirectly via surface light reflections (Mercke et al., 1974b). Using rabbit tracheal epithelial cells, Kennedy and Duckett (1981) confirmed previous observations that ciliary beat frequency (CBF) increases monotonically with increasing
temperature up to 37°C. In human tracheo-bronchial samples, an increase in temperature from 20° to 40°C resulted in a threefold increase in CBF (Konietzko et al., 1981).

**Humidity.** Reductions in relative humidity of air passing over the area of measurement sharply decrease the rate of ciliary beat, especially at high temperature (40°C) (Toremalm et al., 1975).

**Mechanical stimulation.** Cultured ciliated cells of rabbit trachea respond to mechanical stimulation of their cell surface by displaying a rapid transient increase in CBF, dependent on calcium influx (Sanderson & Dirksen, 1986). Both the rise time and response magnitude are influenced by temperature: increased temperature reduces the rise time while simultaneously increasing the response magnitude (Sanderson et al., 1988). Surrounding adjacent and more distal neighbouring ciliated cells display a similar frequency response after a short delay, that is proportional to their distance from the stimulated cell (Sanderson et al., 1988). It has been suggested that the mechanical stimulation of cells results in the formation of an intracellular messenger. This messenger brings about the frequency response in the stimulated cell and simultaneously diffuses into adjacent cells; here the messenger will invoke a frequency response as long as its concentration remains above threshold (Sanderson et al., 1988).

**Ionizing radiation.** Fujiwara and colleagues (1972) reported decreased ciliary activity in tracheal epithelium after irradiation, while Baldetorp and co-workers (1976) and Baldetorp (1985) found increased ciliary
activity during irradiation. All these investigations were performed in vitro and no structural effects with doses higher than 30 Gy were reported. Both fractionated and single dose irradiations have been shown to give dose-dependent physiological and ultrastructural changes in tracheal epithelium (Albertsson et al., 1983, 1984). Low doses of ionizing radiation (2-10 Gy) caused increase in ciliary activity and structural modification in rabbit trachea (Albertsson, 1985). Larger single doses (15-30 Gy) caused reduced and irregular ciliary activity with more marked ultrastructural changes (Albertsson, 1985).

Ionic-strength. Human nasal cilia are most active in iso-osmotic saline [NaCl (9 g/l)] and the activity decreases as ionic strength increases or decreases from that point (Stepper et al., 1965). The CBF of human bronchial cells is well preserved at NaCl concentrations between 5 g/l (80 mmol/l) and 12 g/l (200 mmol/l), but there is rapid loss at concentrations below 0.5 g/l (10 mmol/l) (Luk & Dulfano, 1983).

Viscosity. Increases in viscosity of the medium impair ciliary activity of human nasal (Stepper et al., 1965) and bronchial (Luk & Dulfano, 1983) cells.

pH. Cow tracheal cilia show impairment at pH below 6.7 and above 9.8 with optimal ciliary activity between pH 7.0 and 9.0 (Holma et al., 1977). Ciliary activity of other mammals seems to be similarly affected. Ciliostasis occurs at pH 6.4 in rabbit tracheal cilia, pH 5.2 in rat tracheal cilia and pH 5.8 in guinea-pig tracheal cilia (Wharton, 1931; Negus, 1934; Iravani, 1968). In human bronchial cells, optimal CBF is
elicited at pH 7.0-9.0, with a marked reduction of CBF outside these limits (Luk & Dulfano, 1983).

Inorganic ions. Most studies of the effects of inorganic ions on ciliary beating have been performed in invertebrate systems. Potassium affects the CBF of rat tracheal cells (Melville & Iravani, 1975) and calcium appears to play a key role in the control of ciliary activity (Chapter 9.).

1.7.2. Endogenous mediators

Histamine. In studies in vitro, Wanner and colleagues (1983) found that histamine ($\geq 10^{-4}$ M) caused a slight stimulation (7% increase in CBF) of ciliated epithelial cells obtained from the trachea of sheep, but Scudi and co-workers (1951) reported that histamine ($10^{-3}$ to $10^{-1}$ M) had no effect on ciliary beat measured on guinea-pig and rat tracheal rings. Melville and Iravani (1975), using isolated preparations from rat bronchi, found that histamine did not affect ciliary beat significantly and at high concentrations caused ciliary incoordination. Recently, Bisgaard and Pedersen (1987) reported that histamine did not affect the CBF of human nasal cells.

Serotonin. Serotonin is known to cause a decrease in ciliary activity of rabbit epithelium (Kreuger & Smith, 1960) and an increase in mucus flow in the cat trachea (Dadaian et al., 1971). However, Melville and Iravani (1975) did not found any effect in rat bronchial epithelium.

Bradykinin. Bradykinin ($10^{-7}$ M) causes a rapid, transient increase in CBF in cultured rabbit tracheal explants (Tamaoki et al., 1989a).
**Prostaglandins.** The effects of arachidonic acid metabolites on MCC and CBF have been recently reviewed (Di Benedetto, 1989). Ciliary movement in the frog palate is accelerated by prostaglandins (PG) A₁ and E₁ (Carson & Robbins, 1974). In the same model Maruyama (1984) reported that PGE₁ (2 μM) accelerated the rate of ciliary movement by about 55%, while PGF₂α was without effect. PGE₁ at concentrations between 10⁻⁴ and 10⁻⁶ M, and PGE₂ at concentrations between 10⁻¹⁰ and 10⁻⁶ M caused significant cilio-stimulation in sheep tracheal cells (Wanner et al., 1983). In the same study PGF₂α failed to alter CBF.

**Leukotrienes.** Slow-reacting substances of anaphylaxis (SRS-A) consist of mixtures of leukotrienes (LT) C₄ and D₄ (Lewis et al., 1980). SRS-A liberated in vitro by antigen challenge caused cilio-stimulation (Maurer et al., 1982). LTC₄ (Wanner et al., 1983) and D₄ (Wanner et al., 1986) produced dose-dependent increases in CBF of sheep tracheal cells. It was suggested that LTD₄ increased CBF indirectly via activation of cyclooxygenase (Wanner et al., 1986). However, Bisgaard and Pedersen (1987) reported that LTC₄ and LTD₄ decreased the CBF of human nasal cells.

1.7.3. Neurotransmitters

Using a particle transport method on frog palatine mucosa, Maruyama (1984) reported that low concentrations of noradrenaline have an inhibitory effect, whereas concentration greater than 10⁻⁷ M induced the release of a PG-like substance which accelerated ciliary movement.
Adrenaline and acetylcholine were found to be cilio-excitatory in rat epithelium (Melville & Iravani, 1975). In the frog palate preparation ciliary movement was suppressed by dopamine and was accelerated by 6-hydroxydopamine (Maruyama et al., 1983), which depletes dopamine from the vesicles (Hokfelt & Ungerstedt, 1973).

1.7.4. Therapeutic agents

Anaesthetics. In 1958, measuring the speed of rotation of tracheal explants, Corssen and Allen reported cilio-inhibition by lignocaine. The finding was later confirmed in ferret tracheal rings (Manawadu et al., 1978; Mostow et al., 1979) and in human nasal cells (Rutland et al., 1981). However, Dudley and Cherry (1978) found that concentrations of lignocaine which were ciliostatic in vitro did not impair ciliary activity when administered in vivo. Rutland and colleagues (1981) also did not find cilio-depression in vitro following in vivo administration of lignocaine. The effects of opiates on ciliary activity have been less well studied. Iravani and Melville (1976) reported in vitro cilio-inhibition in the rat after administration of codeine, but morphine had no effect. In the same preparation phenobarbital caused cilio-stimulation at low concentrations and cilio-inhibition at high doses (Melville & Iravani, 1975).

Mucolytics and expectorants. Potassium iodide and ammonium chloride are cilio-stimulatory in rat epithelium (Melville & Iravani, 1975). Ambroxol is the eight metabolite of bromhexine and it is claimed to have
mucolytic properties (Pavia, 1984). Ambroxol significantly stimulates the CBF of isolated cells from guinea-pig tracheas (Disse & Ziegler, 1987). Compounds with free sulphydryl groups such as S-carboxymethylcysteine and acetylcysteine are believed to be able to split the disulphide bonds of the long glycoproteins of mucus and thereby reduce its viscosity (Sheffner, 1963; Marriott & Richards, 1974). Furthermore, such compounds may also reduce the 'compactness' of the fibrillar structure of the mucus by replacing fucomucins by sialomucins (Havez et al., 1970). In several animal studies (Melville & Iravani, 1975; Dudley & Cherry, 1977; Iravani et al., 1978; Dreisin & Mostow, 1979; Yanaura et al., 1981a; Roomans et al., 1983) it has been found that high concentrations of N-acetylcysteine (NAC) decrease CBF and that this effect, related to its sulphydryl group, is only partly reversible (Dreisin & Mostow, 1979). There have been two reports that low concentrations of NAC stimulate CBF, suggesting that this may be one of the beneficial effects of NAC in vivo (Iravani et al., 1978; Yanaura et al., 1981a). NAC has been also found to decrease CBF in human bronchial (Low et al., 1985) and nasal (Stafanger et al., 1987) cells in vitro, but the concentrations were effective above 10 mg/ml and 2 mg/ml respectively, and this is far more than the concentrations found in vivo in secretions following oral administration by Rodenstein and colleagues (1978).

_Beta agonists and antagonists._ Adrenergic drugs in addition to being effective bronchodilators might have a further role to play in the control of mucus transport in the airways in obstructive lung disease. Such
drugs have attracted the interest of many investigators and their effects on CBF, mucus production and transport have been widely reported (Wanner, 1981). Adrenergic agents increase whole lung clearance and tracheal mucous velocity in healthy subjects and in patients with chronic obstructive airways disease and cystic fibrosis (Santa Cruz et al., 1974; Wood et al., 1975; Yeates et al., 1975; Foster et al., 1976). Isoprenaline, a non selective β-adrenoceptor agonist, has been found to increase CBF in cultured rabbit tracheal cells (Verdugo et al., 1980; Sanderson & Dirksen, 1989) and this effect was inhibited by the non-selective β-adrenoceptor blocker propanolol (Verdugo et al., 1980). Isoprenaline also increases the CBF of rat (Lopez-Vidriero et al., 1985) and canine (Yanaura et al., 1981b) tracheal cells. A 70% increase of CBF was observed in a rat whole lung preparation with salbutamol (Van As, 1974), which mainly acts on β2-adrenoceptors. A stimulatory effect of salbutamol has been also reported in studies on canine tracheal cells (Yanaura et al., 1981b) and isolated cells from guinea-pig tracheas (Disse & Ziegler, 1987). Orciprenaline is cilio-excitatory in rat epithelium (Melville & Iravani, 1975). Hesse and colleagues (1981) found that 10⁻⁵ g/ml of reproterol caused a mean increase in human bronchial CBF of 16% and 10⁻⁴ M terbutaline was found to stimulate CBF in bronchial biopsies by 40% (Clarke & Lopez-Vidriero, 1983). Similarly, terbutaline was cilio-stimulatory on sheep tracheal cells in vitro (Maurer et al., 1982). Fenoterol (10⁻⁷ M) increased CBF of bovine tracheal cells in vitro and the effect was larger when the β₂ agonist was applied to the serosal surface.
(Wong et al., 1988a). The same group, using a new technique for measuring CBF in vivo, found that aerosolized fenoterol (10^{-6} \text{ M}) stimulated CBF in intact, anaesthetized beagles (Wong et al., 1988b). Beta-adrenergic blocking agents slow CBF (van de Donk & Merkhus, 1982). Propanolol by itself does not affect ciliary activity at low concentrations, but at high concentrations (10^{-4} \text{ M}) it produces a decrease of CBF (Verdugo et al., 1980). This cilio-inhibitory effect could be dependent on the local anaesthetic properties of this drug, as anaesthetic effects have been shown to appear with high concentrations of propanolol (Hermansen, 1969).

Cholinergics and anticholinergics. Both in vivo and in vitro experiments on animals have shown a stimulatory effect of cholinergic compounds on mucociliary transport (Pavia, 1984). Cholinergic agents increase mucus secretion from submucosal glands in human bronchial tissue culture (Sturgess & Reid, 1972) and, at high concentrations, increase the speed of rotation of bronchial explants (Corrsen & Allen, 1959). Both these effects are blocked by atropine. Pilocarpine is cilio-excitatory in rat epithelium (Melville & Iravani, 1975) and methacholine increases CBF of sheep tracheal cells in vitro (Maurer et al., 1982). Methacholine (10^{-7} \text{ M}) also increases CBF of bovine tracheal cells in vitro and the effect is larger when the muscarinic cholinergic agonist is applied to the mucosal surface (Wong et al., 1988a). Aerosolized methacholine stimulates CBF in intact, anaesthetized dogs (Wong et al., 1988b). Atropine slows CBF in vitro, but only at very high concentrations.
(10⁻⁵ g/ml) (Iravani, 1972), possibly after an initial period of cilio-stimulation (Corssen & Allen, 1959).

**Corticosteroids.** The precise pharmacological action of corticosteroids in asthma is not well defined. It is possible that one of the mechanisms might be the enhancement of MCC thereby reducing the tendency to mucus plugging in the small airways (Pavia et al., 1983). Prednisolone is cilio-excitatory in rat epithelium (Melville & Iravani, 1975), but beclomethasone dipropionate and flunisolide cause a dose-related decrease in CBF of human nasal cells (Stafanger, 1987).

**Xanthines.** Aminophylline is cilio-excitatory in rat epithelium (Melville & Iravani, 1975). Theophylline increases the CBF of canine tracheal cells in doses of 10⁻⁵ M and above (Yanaura et al., 1981b).

**Preservatives.** Methylparaben is a potent inhibitor of ciliary activity (Mostow et al., 1979). Lipophilic compounds like chlorbutol, chlorocresol and the p-hydroxybenzoates cause a strong decrease of CBF in the chicken embryo trachea (van de Donk et al., 1980). The effects are reversible when the exposure time is limited. In the same model, more polar substances like the quaternary ammonium compounds (benzalkonium chloride, domiphen bromide) and chlorhexidine gluconate decrease the frequency more slowly, but the effects are not reversible. The mercuric compounds like thiomersal and phenylmercuric borate decrease the frequency severely and the effects are not reversible. Benzalkonium chloride and thiomersal are ciliotoxic on human nasal epithelium *in vitro* (Stanley et al., 1985) and propylene glycol inhibits the
CBF of human nasal cells in vitro (Stafanger, 1987).

Ethyl alcohol. MCC is frequently impaired in patients with chronic alcoholism (Venizeles et al., 1980). Maurer and Liebman (1988) reported inhibition of ciliary motility at very high ethanol levels (2%) on cells obtained by brushing the trachea of sheep, but observed no acute impairment of ciliary function at ethanol concentrations comparable to those achieved from social drinking (0.5-1%). Indeed, they found an unexpected stimulation of ciliary beating at low levels of ethanol (0.01-0.1%).

1.7.5. Environmental pollutants

Smoking. The adverse effects of tobacco smoke on MCC and ciliary beating have been known for many years (Sleigh, 1984; Di Benedetto, 1990). Compounds such as hydrogen cyanide, acrolein, formaldehyde, ammonia, and phenols in tobacco smoke are toxic to mammalian cilia in vitro (Kensler & Battista, 1963) and small quantities of whole smoke or its aqueous extract cause ciliostasis in human respiratory epithelium in vitro (Dalhamn, 1959; Ballenger, 1960). However, there is no significant difference between the CBF in vitro of smokers and non-smokers (Yager et al., 1980; Konietzko et al., 1981; Dulfano et al., 1981; Stanley et al., 1986).

Sulphur dioxide. Sulphur dioxide (SO₂) is a major component of urban pollution whose detrimental effects on pulmonary function have been studied (Wanner, 1977). In the rat it may cause reduction of mucus
transport and in some cases reduction of ciliary beating (Dalhamn, 1956). In sheep, a combination of low level ozone and SO$_2$ causes depression in tracheal mucous velocity but not in CBF (Abraham et al., 1986).

1.7.6. Cellular and bacterial products

Lysozyme is an important resistance factor in the respiratory defence mechanism. Recent results indicate that egg-white lysozyme increases the CBF of human nasal cells (Hisamatsu et al., 1986).

Many reports have documented the presence of factors in serum and from leucocytes that have cilio-inhibitory activities. Serum from patients with cystic fibrosis is toxic to a variety of in vitro ciliated systems, including oyster gill (Bowman et al., 1969) and rabbit trachea (Spock et al., 1967). A low molecular weight glycoprotein (or family of proteins) was purified from the serum (Blitzer & Shapira, 1982), plasma (Carson & Bowman, 1982), and lymphocytes or mononuclear leucocytes of patients with cystic fibrosis, asthma and in other patient groups (Conover et al., 1973; Wilson & Fudenberg, 1978; Wilson, 1983), but not in normal subjects. Most of these factors appear to produce ciliary dyskinesia when tested on rabbit trachea (Wilson & Fudenberg, 1978; Blitzer & Shapira, 1982; Rutland et al., 1983; Wilson, 1983). However, the in vitro CBF of nasal epithelium is normal in patients with cystic fibrosis (Rutland & Cole, 1981; Rossman et al., 1984) and is not slowed following incubation with homologous serum, nor is there any effect of cystic fibrosis serum on normal human nasal or bronchial ciliated epithelium (Rutland et al.,
1983). This interspecies variation is also true for the ciliotoxic effect of serum from patients with acute asthma (Greenstone et al., 1984). Patients with bronchiectasis have a serine protease, possibly neutrophil elastase, found in sputa that reduces CBF both of rabbit (Tegner et al., 1979) and human (Smallman et al., 1984) ciliated cells. Sputum from patients with chronic bronchial asthma during clinical exacerbations is ciliostatic in the frog palate (Dulfano et al., 1982) and human bronchial mucosa (Dulfano & Luk, 1982). Frigas and colleagues (1980, 1981), pursuing long-term studies with a major basic protein (MBP) isolated from eosinophils of asthmatic patients, found it capable of inducing ciliostasis in man and pig. Elevated levels of eosinophil MBP have been found in the sputa of patients with bronchial asthma (Frigas et al., 1981; Dor et al., 1984) and purified human eosinophil MBP has been shown to be cilio-inhibitor in rabbit tracheal explants (Hastie et al., 1987).

MCC of the upper and lower respiratory tracts is delayed in conditions where bacterial infection is present and purulent secretions are produced (Wilson, 1988). Bacterial products may delay clearance by their effect on cilia. Several bacteria and bacterial products have been shown to produce factors which slow ciliary beating and damage the epithelium. Among these are *Haemophilus influenzae* (Denny, 1974; Wilson et al., 1985; Johnson & Inzana, 1986), *Pseudomonas aeruginosa* (Reimer et al., 1980; Hingley et al., 1986ab; Wilson et al., 1987), *Corynebacterium diphtheriae* (Baseman & Collier, 1974), *Mycoplasma pneumoniae* (Collier & Clyde, 1971; Hu et al., 1976; Chandler & Barile, 1980), *Mycoplasma*

1.8. Measurement of ciliary activity

The development of methods to record ciliary movements objectively and quantitatively has been difficult. Earlier attempts to assess ciliary activity indirectly were based on measurements of transport of particles (seeds or charcoal powder) over ciliated cells (Sharpey, 1835), or else on the rotation of spheres or spindles suspended over the surface of ciliated cells (Engelmann, 1868, 1877; Inchley, 1921). The speed of rotation of bronchial explants has been used as an indirect measure of ciliary beating, which proved valuable in the investigation of drug toxicity (Corrsen & Allen, 1958).

The methods available for the registration of ciliary activity can be divided into three basic categories:

1. stroboscopic techniques;
2. cinematographic methods;
3. transmitted light techniques.

Strobotachimetry is one of the earlier methods used and is based on the adjustment of stroboscopic flash frequency with that of the light
reflected from the beating cilia (Gray, 1930; Lucas, 1931; Adalis et al., 1978). The results are immediately available, but the ciliary movements are not exactly periodic, so it is difficult to obtain the "freeze" of movements. Moreover, the rate of flashes is disagreeable to the operator and can cause epileptogenic effects (Braga et al., 1986). The stroboscopic method may be feasible when working with inferior animals such as mussels, where every cilium can be distinguished and the frequency is low (Jennison & Bunker, 1934). It is not feasible when studying mammalian cilia (Dalhamn, 1970).

The use of high-speed cinematography was first described by Proetz (1932) and further developed by Dalhamn (1955, 1960) and others (Wilson et al., 1975; Aiello & Sleigh, 1977; Cheung & Jahn, 1976; Sanderson & Sleigh, 1981). These methods allow the assessment of all the parameters of the ciliary cycle, namely frequency, amplitude, and phase coherency of ciliary beat, as well as the dynamics of bend propagation along the ciliary shaft. High-speed cinematography is a high-speed motion picture recording that needs a special camera with frame rate of up to 500-700 frames/sec. After developing, a repeated viewing at low speed (2-24 frames/sec) gives the possibility of counting by direct vision the flickering activity (Dalhamn, 1955, 1960) or the number of ciliary beats (Proetz, 1932; Wilson et al., 1975; Cheung & Jahn, 1976). This method with the use of a very precise time marker is accurate. However, it is expensive, the developing is laborious and there is a time lag between the filming and the data collection from the developed film.
More recently, videotape records have been used to overcome the inconvenience and expense associated with the use of film records (Rossman et al., 1980; Maurer et al., 1982; Dresdner & Wong, 1984; Morgan et al., 1984). In these studies the records are played back slowly, while an observer counts the number of ciliary beat cycles occurring in a short time interval. To overcome observer interpretation, an automated method has been developed, that applies computer-implemented algorithms to digitized video-image sequences of ciliary activity (Hennessy et al., 1986).

In 1952, Dalhamn and Rylander developed an ingenious photometric method based on measuring the light intensity modulation produced by the moving cilia when they are transilluminated on the stage of a microscope. Although this method has been reported to be unreliable when ciliary movement becomes asynchronous (Naitoh & Kaneko, 1973), it has found wide application in *in vitro* studies. In its various forms, this is the most widely used technique for measuring CBF. In principle the technique allows a photosensitive cell to detect light variations produced by the movement of cilia. The changes in light intensity thus produced can be converted to a voltage signal. A number of workers use a photomultiplier to detect and enhance the light signal (Yager et al., 1978; Rutland et al., 1982b). The technique is very simple and, when combined with spectral analysis, provides direct recordings of the CBF (Kennedy & Duckett, 1981; Puchelle et al., 1982; Eshel et al., 1985). A photoelectric technique based on simultaneous measurement of scattered
light from two points on the ciliary epithelium has been used to measure *in vitro* the phase differences between beating cilia and, therefore, the wave-length of the metachronal wave (Eshel & Priel, 1987). The various transmitted light techniques appear to give reproducible results, are convenient to use and require minimal subjective assessment. Their disadvantages are primarily related to cost and lack of durability of photomultipliers. Flash saturation of a photomultiplier with excess light can lead to a variable recovery time of the system and in the extreme will permanently damage the tube (Teichtahl et al., 1986). Other potential disadvantages are detection of extraneous vibrations and the inability of some systems to detect light changes produced by a single cilium or even cilia from one cell. The results would then be an average or mass effect CBF from a number of ciliated cells.

Different methods have been developed that combine video-recording and transmitted light techniques. Kennedy and Ranyard (1983) have used a photomultiplier to detect the *in vitro* CBF of rabbit tracheal cells displayed on a video monitor. Braga and colleagues (1986) used an instrument with a digital readout purposely designed to receive and count signals from a phototransistor placed directly on the screen of a TV monitor. Teichtahl and co-workers (1986) have developed a modification of the transmitted light technique in which ciliated cells are displayed on a video unit and the light variations from beating cilia are detected by an inexpensive and durable photodiode. Recently, an automated computer-assisted image-analysis system to examine high-
speed films of beating cells has been developed (Sanderson et al., 1988): the method simulates a multisensor system for measuring CBF of many cells simultaneously, enhancing the photoelectronic technique with a single detector previously used by the same group (Sanderson & Dirksen, 1985).

A technological innovation to study ciliary activity is the application of dynamic laser-scattering spectroscopy to detect the movement of cilia (Lee & Verdugo, 1976, 1977). Dynamic laser spectroscopy had been successfully implemented in the past in a broad range of applications to detect molecular motion (Pecora, 1964; Dubin et al., 1967), sperm motility (Berge* et al., 1967) and bacterial movement (Nossal & Chen, 1972). This technique is based on the assessment of the Doppler-shift induced in the laser light by moving scatterers and, in the case of ciliary motion \textit{in vitro}, it has been shown to provide highly accurate measurements of the frequency of cilia beat in reference to high-speed cinematography (Lee & Verdugo, 1976, 1977). However, it is very complicated in its devices and in the data extraction.

New techniques for measuring ciliary activity \textit{in vivo} are under study at the very present. Verdugo and Golborne (1988) have measured the CBF using a fibre optic laser spectrometer in tracheae of dogs and oviducts of rabbits. The instrument is an extension of the previous application of dynamic laser spectroscopy to detect ciliary activity \textit{in vitro} (Lee & Verdugo, 1976, 1977). Recently, another method for measuring CBF \textit{in vivo} has been developed (Wong et al., 1988b). The technique uses
a heterodyne mode correlation analysis laser light-scattering (CALLS) system. The CALLS system derives the periodicity of the ciliary beat from the Doppler-shift of randomly depolarized, backscattered photons from the cilia, a measurement not affected by the coupling mucous layer.
2. APPARATUS AND TECHNIQUES

2.1. Collection of the samples

*Rat tracheal epithelium.* Sprague-Dawley rats were killed by a blow to the head. The trachea was immediately removed and washed several times in physiological saline solution. The external surface was cleared of fat and connective tissue. The laryngeal segment, which contains almost all the submucosal glands, and the upper third of the trachea were discarded. The rest of the trachea was sectioned into small rings (1 mm thick) and washed several times with tissue culture medium (1X Medium 199 modified with Earle’s Salts and 2.2 g/l NaHCO₃ without L-glutamine, Flow Laboratories).

*Human nasal epithelial biopsies.* Different techniques can be used to obtain ciliated cells from the nasal mucosa. Samples can be collected with a curette (Pedersen et al., 1983; Duchateau et al., 1985) and can also be harvested with the use of an operating microscope (Douglas et al., 1968). An alternative approach to a curette is the use of cytological brushes (Yager et al., 1978; Konietzko et al., 1981; Carson et al., 1985).

We obtained nasal ciliated epithelium using the brushing technique described by Rutland and Cole (1980). An auriscope was introduced into one nostril to allow the inferior nasal turbinate to be seen. A cytology brush 2 mm in diameter was inserted through the auriscope between the inferior turbinate and lateral nasal wall, quickly moved posteriorly for about 1 cm along the mucosal surface, and then moved anteriorly as it
was removed. Cellular material adhering to the brush was dislodged by brisk agitation in Eppendorf tubes containing Medium 199. Moistening the brush with the culture medium before use increased the amount of tissue obtained (Rutland & Cole, 1980). Tissue yield was also increased by having the subject blow his nose to clear excess secretions (Rutland et al., 1982a). The procedure could be repeated immediately from the same or another site in the same area until sufficient tissue was visible in the tube. Two or three brushings were frequently taken from the same nostril. Occasionally a small amount of surface bleeding occurred in inflamed mucosa in which case the controlateral nasal cavity was sampled. Subjects acceptance has been good and repeat studies have been performed in many instances. Over 300 brushings have been performed without significant morbidity (about 20 minor nose bleeds). Among the adult subjects, less than 10% complained of pain, while most described sensations of a tickle or a desire to sneeze.

*Human endobronchial epithelial biopsies.* Endobronchial epithelial biopsies were obtained during fiberoptic bronchoscopy. All subjects were undergoing bronchoscopy for diagnosis of a variety of pulmonary problems, suspicion of bronchial carcinoma being the commonest. Endobronchial brush biopsy specimens were obtained by passing a nylon brush through the biopsy channel of the fiberoptic bronchoscope and scraping the epithelium in an area that appeared free from inflammation. Specimens were then dislodged into Eppendorf tubes containing Medium 199.
2.2. Storage of the samples

Most of the experiments were performed within one hour of the collection of the samples. The storage procedure was standardized as follows.

When the experiments were performed within one hour of the time of collection, the samples were kept at room temperature in Eppendorf tubes containing 1 ml of Medium 199.

If the study was performed between one hour and eight hours of the collection, the samples were kept at 4°C in Eppendorf tubes containing 1 ml of Medium 199. In this occurrence the culture medium was replaced with fresh medium after the first four hours.

When the experiments were performed between eight hours and three days from the time of collection, the samples were cultured. The cells were allowed to settle to the bottom of the Eppendorf tube on ice for 20 minutes. The supernatant was then removed and the cells were washed by resuspension in 1.5 ml of a solution of Medium 199 supplemented with Gentamicin (25 μg/ml), Amphotericin B (25 μg/ml), Streptomycin (100 μg/ml) and Penicillin (100 U/ml), followed by centrifugation at 20 x g. The 'wash' solution was then removed and the cells were resuspended in 3 ml of the above solution supplemented with 1% foetal calf serum, transferred to Petri dishes (diameter 5 cm) and placed in an incubator (37°C, 5% CO₂).

If the storage period was more than three days, the samples were preserved in liquid nitrogen at -196°C, following the cryopreservation
technique described in Chapter 5.

2.3. Microscope preparations

*Sealed coverslip microscope slide preparations.* Microscope slide preparations for measuring CBF were made by applying a thin layer of silicon grease (RS Components Ltd.) around the edges of a coverslip (Chance Propper, 22 x 22 mm) by means of a syringe attached to a modified intravenous cannula. Fragments of epithelium were transferred from the culture medium with a Pasteur pipette and deposited over the coverslip. A standard microscope slide (Chance Propper) gently placed over the coverslip sealed the preparation, which could then be inverted and examined microscopically, coverslip upwards.

*Perfusion preparation.* For studies in perfusion the samples were mounted in a tissue culture chamber (Prior U.K., modified) with an internal volume of 0.35 ml. A picture of this chamber is shown in Figure 2.1. The chamber consisted of the following parts:

1. the bottom part of the aluminium housing, bearing two holes on opposite sides for in- and out-puts of solutions;
2. two glass coverslips, 32 mm in diameter and thickness No. 1 (Chance Propper);
3. a silicone rubber ring, 3 mm thick, placed between the coverslips. The ring was cut at one side to obtain a C-shape;
4. a plastic plate inserted inside the rubber ring to reduce the height of the chamber;
Fig. 2.1. Chamber for perfusion studies.
(5) the top part of the aluminium housing, which compressed the coverslips against the rubber ring and held them tightened on the bottom part of the housing by three screws;

(6) one disposable vein infusion set (Butterfly-21, Abbot Laboratories), used for perfusing solutions into the chamber. This set was composed of 19.1 x 0.6 mm hypodermic needle with a 305 mm extension tube.

(7) the solutions were introduced into the chamber by using a 5 ml syringe inserted on an infusion pump (Moleculex, Thomae).

A 32 mm coverslip was placed at the bottom of the chamber. The silicone rubber ring, pinched on one side by the needle and cut on the exactly opposite side was placed on the lower coverslip. The plastic plate was inserted inside the rubber ring. A drop of culture medium was deposited on the centre of the plastic plate and the explant was immersed in this drop. The upper cover-slip was next fitted on the rubber and the top part of the chamber was screwed on the bottom part. The infusion set was attached to the perfusing syringe and the solutions were introduced into the chamber.

The out-put system consisted of a plastic tube with a two-way system that allowed the discharge of the solutions and the insertion of a thermometer microprobe, for continuous monitoring of the temperature in the chamber.
2.4. Measurement of ciliary beat frequency

2.4.1. Cell selection

In all studies, the choice of the cells from which the ciliary beat frequency (CBF) was measured was randomized by using an eyepiece graticule. Bias was avoided by choosing cells laying closest to the crosspiece of the graticule on the equatorial line and which fulfilled the following criteria (Lopez-Vidriero & Clarke, 1982): 1) the cilia were beating perpendicularly to the light source, and 2) the ciliated cells were fixed in a cluster of tissue or at their basement membrane (Deitmer, 1986).

2.4.2. Photometric technique

We measured CBF photometrically (Fig. 2.2.). The sample preparation was placed on the stage of a phase-contrast microscope, fitted with a photomultiplier connected to an amplifier. Light directed from below and passing through the specimen was deflected by the sweeping action of the cilia. Variations in light intensity were thus produced corresponding to the beat frequency. The light variations were detected by the photomultiplier, transduced to electric impulses and amplified. The electrical signal was fed through a lowpass filter into: a) an ultra-violet oscillograph, which provided a permanent record of ciliary activity; b) a storage oscilloscope screen. The transduced electrical signal was also analyzed by a Spectrum Analyzer, from which the frequency could be
Fig. 2.2. Basic equipment for measuring ciliary beat frequency. microscope (a), photomultiplier (b), amplifier (c), ultra-violet oscillograph (d), storage oscilloscope (e), spectrum analyzer (f), oscilloscope connected to the spectrum analyzer (g).
2.4.3. Microscope and photometer

A phase-contrast microscope (Leitz Dialux 20, W. Germany) was used. Strips of epithelium with beating cilia were easily seen at a magnification of 320 (Objective: Long W/D L32/0.40) by bright-field illumination with the condenser (SK standard condenser, condenser top 0.55 S 15) at a low aperture.

A Leitz MPV compact microscope photometer transduced light intensity into an electrical signal. The photometer was set up in a room that met the following conditions: a) constant temperature, b) black-out facility, c) good ventilation, d) freedom from dust. Fluctuations of the mains current of more than \( \pm 10\% \) were avoided (they readily occur when instruments or machines of high current consumption are operated in the neighbourhood of the photometer). The instrument table was rigid.

The photometric device consisted of: a) a stabilized light source, b) insertable field diaphragms, c) a binocular tube with vertical observation tube to accept d) the photometer attachment with photomultiplier, e) the insertable measuring diaphragms, f) a control panel, g) the digital display unit, h) the power units for stabilizing the light sources. An essential requirement for photometry of microscopic specimens is a stabilized light source, whose spectral characteristics allow the isolation of monochromatic light of sufficient intensity. Our light source was a 12 v 100 W tungsten halogen lamp. Illumination of the photometer field
diaphragm allowed a small rectangular area (approximately 1.5 x 5 μm) to be orientated in the long axis of the cilia so that only few cilia beat across this area, intermittently preventing light from reaching the photometer.

2.4.4. Reading the signal

The transduced electrical signal was amplified and filtered by a lowpass filter with a cut-off above 40 Hz. Finally, this signal was led to three reading devices.

An ultra-violet oscillograph recorder (SE Labs 6150) provided a permanent record of ciliary activity. From waveforms written on an oscillogram, the number of ciliary beats per second was counted (Fig. 2.3.).

The signal was also displayed on a storage oscilloscope screen (Telequipment TD 51). We used this real time reading to check the quality of the signal before recording with the oscillograph.

Finally, the signal was led to a Spectrum Analyzer (Medical Electronics, Royal Free Hospital) connected to another oscilloscope (Telequipment DM 63). By utilizing the Spectrum Analyzer it was possible to identify the dominant frequency present in a 10-second analog signal (Fig. 2.4.).
Fig. 2.3. Signal recorded by the oscillograph. The bar represents 10 cm.

Ciliary beat frequency = 8 Hz (paper speed 10 cm/sec).
Fig. 2.4. Signal obtained with the Spectrum Analyzer. Ciliary beat frequency = 12 Hz (the arrow indicates the 10 Hz line on the oscilloscope screen).
2.4.5. Validation of the Spectrum Analyzer

In order to validate our device for spectrum analysis of the frequencies, we compared data obtained with the oscillograph and the Spectrum Analyzer.

The CBF of 20 randomly selected areas of rat tracheal rings (see page 49) was measured. Two independent observers reported the Spectrum Analyzer data displayed on the screen of the oscilloscope and, at the same time, 10 seconds of oscillograph recording were taken. The oscillograms were coded and analyzed blind by the two observers. Finally, the data were compared. The results showed strong intra-observer correlations between data obtained with the two devices (r=0.978; r=0.955) (Fig. 2.5.). Also strong inter-observer correlations were found with both the oscillograph and the Spectrum Analyzer (r=0.968; r=0.980) (Fig. 2.6.).

2.4.6. Temperature control

With our equipment it was possible to perform studies on ciliary activity both at room temperature and at 37°C.

When studies were performed using sealed coverslip microscope slide preparations (see page 52), the temperature control was easily obtained by using a warm stage (Microtec, Micro Instruments). The slide preparation was placed on the stage heating unit that was designed to fit the microscope stage. The warm stage was a fully automatic unit designed to operate in a temperature range from ambient to 60°C. It was
Fig. 2.5. Comparison between oscillograph (UV) and Spectrum Analyzer (SA) data (frequency expressed in Hz) recorded by two independent observers (A and B).
Fig. 2.6. Comparison between readings (frequency expressed in Hz) by two observers (obs. 1 and 2) of the oscillograph (UV) and Spectrum Analyzer (SA) data.
preset to the temperature required which was then maintained automatically.

When studies were performed using chamber preparations, the temperature control was a more difficult issue. The problem was to maintain at a constant temperature not only the chamber, but also the solutions perfusing the chamber itself. Because of the dimension and the thermal conductivity of the chamber, the warm stage was not sufficient to maintain the sample at 37°C. We, therefore, designed an apparatus to ensure a constant temperature of 37°C during perfusion studies. We used (Fig. 2.7.):

(1) a wood box covering the microscope;
(2) a plastic box covering the infusion pump;
(3) an air curtain blowing warm air in both boxes;
(4) the warm stage;
(5) a digital thermometer.

The two boxes were tightly connected and had holes for the air curtain tubes and for access to the syringe and to the microscope knobs. Our procedure in studies performed at 37°C can be summarized as follows:

(a) switch on the air curtain set at 40°C for 15 minutes before the experiment;

(b) switch on the warm stage set at 38°C until we detected 37°C in the chamber and then decrease the setting to 37°C and after 5 minutes to 36°C;
Fig. 2.7. Complete equipment for measuring ciliary beat frequency. Perfusion pump (a), boxes (b,c), air curtain control unit (d), warm stage control unit (e), digital thermometer (f).
(c) perfuse with solutions warmed up to 37°C for 30 minutes in an incubator before the experiment.

The temperature was continuously monitored by using a digital thermometer (Comark Electronics Limited) with the microprobe in contact with the solution in the chamber (Fig. 2.8.). If little variations of the temperature were detected during the experiment, it was possible to adjust the warm stage setting.
Fig. 2.8. Temperature in the chamber during perfusion studies. The arrows indicate the beginning and the end of a period of perfusion.
3. VARIABILITY OF HUMAN CILIARY BEAT FREQUENCY IN VITRO

3.1. Summary

This study quantified the \textit{in vitro} intra-cell, intra-sample and inter-sample variability of ciliary beat frequency (CBF) in human nasal epithelium. Samples of ciliated epithelium were obtained by brushing the inferior turbinates of 10 healthy subjects. Aliquots from each sample were transferred to sealed coverslip microscope slide preparations and the mean intra-sample CBF was obtained by taking measurements from 20 randomly selected cells. The remaining aliquots were mounted in a chamber and perfused continuously with culture medium at a flow-rate of 0.02 ml/min. Using this preparation, we monitored the CBF of a single cell for 10 seconds every 5 minutes for 1 hour. The results revealed mean intra-cell CBF ± SEM of 7.3 ± 0.4 at room temperature with a coefficient of variation of the frequency (CoV) of 5%, and 11.9 ± 0.4 (CoV = 4%) at 37°C. The mean intra-sample CBF was 7.6 ± 0.2 (CoV = 18%) at room temperature and 12.8 ± 0.4 (CoV = 17%) at 37°C. The inter-sample mean CoV was 7% at room temperature and 11% at 37°C. Our findings can be used to assess the significance of results in studies on the effects of cilio-active agents on CBF.
3.2. Introduction

Different techniques are used to investigate the in vitro effects of cilio-active drugs and cilio-modulating agents. The methods available at present can be divided into two basic categories: 1) techniques using microscope slide preparations (Rutland et al., 1981; Stanley et al., 1986; Wilson et al., 1987; Han et al., 1990) and 2) methods using perfusion chambers (Eckert & Murakami, 1972; Verdugo, 1980; Maurer et al., 1982; Ohashi et al., 1983; Wanner et al., 1983; Pedersen, 1983; Roth et al., 1985; Lopez-Vidriero et al., 1985; Stafanger, 1987).

When a sealed coverslip microscope slide preparation is used, the sample of ciliated epithelium is divided into aliquots and each aliquot is then mixed with a control solution and with one or more concentrations of the substance under investigation. Results are obtained by comparing the mean ciliary beat frequency (CBF) values for each slide preparation, calculated by taking a chosen number of measurements on cells randomly selected in the microscope field. In this type of studies the intra-sample variability of the CBF is critical in order to detect correct results.

The advantage of using a perfusion chamber preparation is that the ciliated cells can be continuously observed under a range of conditions. Changes in CBF due to the effect of the perfusate can be detected from the same cell observed in real time. In these studies the intra-cell variability of the CBF is critical in order to obtain reliable information.

In order to quantify the above parameters of variability we
investigated the *in vitro* intra-cell, intra-sample and inter-sample variability of CBF of human nasal epithelium.

3.3. Materials and methods

*Sample collection.* The material for this study was obtained from 10 healthy volunteers aged 26 to 38 years (mean 30.2 years). The group included 7 men and 3 women; 5 of them were smokers. None of them reported previous history of upper respiratory tract infections for at least one month. Samples of nasal ciliated epithelium were obtained by brushing the inferior turbinates (see page 49).

*Experimental protocol.* Adherent epithelium was dislodged from the brush by brisk agitation in a 5 ml plastic bijoux containing 2 ml of culture medium (Medium 199, Flow Laboratories). Each sample was then divided into four aliquots. Two aliquots were transferred to sealed microscope coverslip slide preparations (see page 52) and the remaining were mounted in a tissue culture chamber (see page 52), where they were perfused continuously with Medium 199 at a flow-rate of 0.02 ml/min. With the slide preparations we measured the mean intra-sample CBF at room temperature (22.6 ± 0.9°C) and at 37°C. Each value represents the mean of CBF measurements taken from 20 cells chosen at random (see page 55) and situated in different clumps. The chamber preparation was used to monitor the CBF of a single cell chosen at random (see page 55) for a period of 1 hour at room temperature (23.0 ± 0.6°C) and at 37°C. The spontaneous variation of the CBF in the
chamber experiments was obtained by taking CBF measurements for 10 seconds every 5 minutes for 1 hour, the microscope light being switched off between measurements.

**Statistical analysis.** CBF values were expressed as mean ± SEM. Variability was expressed in terms of the coefficient of variation (CoV), defined as the ratio of the standard deviation to the mean value as a percentage. Statistical differences between CoV were determined by a Mann-Whitney U test. Significance was accepted when $P < 0.05$.

3.4. Results

The experimental results revealed a mean ± SEM CBF for the same cell observed for 1 hour of 7.3 ± 0.4 at room temperature and of 11.9 ± 0.4 at 37°C (Tab. 3.1.). The mean CoV were 5% and 4% respectively.

The mean ± SEM intra-sample CBF was 7.6 ± 0.2 at room temperature and 12.8 ± 0.4 at 37°C (Tab. 3.2.). The mean CoV were 18% and 17% respectively.

The mean intra-sample CoV of the frequency was significantly ($P < 0.01$) higher than the intra-cell CoV, both at room temperature (Tab. 3.3.) and at 37°C (Tab. 3.4.).

The inter-sample mean CoV was 7% at room temperature and 11% at 37°C.
Tab. 3.1. Mean intra-cell ciliary beat frequency (Hz)* during 1 hour of observation (readings for 10 seconds every 5 minutes).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Room temperature</th>
<th>37 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.4 (0.2)</td>
<td>11.2 (0.5)</td>
</tr>
<tr>
<td>2</td>
<td>6.9 (0.1)</td>
<td>12.4 (1.1)</td>
</tr>
<tr>
<td>3</td>
<td>8.2 (0.7)</td>
<td>11.7 (0.3)</td>
</tr>
<tr>
<td>4</td>
<td>5.9 (0.2)</td>
<td>12.0 (0.2)</td>
</tr>
<tr>
<td>5</td>
<td>9.2 (0.4)</td>
<td>13.7 (0.4)</td>
</tr>
<tr>
<td>6</td>
<td>8.1 (0.3)</td>
<td>9.3 (0.1)</td>
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<tr>
<td>7</td>
<td>5.4 (0.3)</td>
<td>11.0 (0.3)</td>
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<td>8</td>
<td>6.6 (0.1)</td>
<td>12.9 (0.9)</td>
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<tr>
<td>9</td>
<td>6.1 (0.5)</td>
<td>12.2 (0.5)</td>
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<tr>
<td>10</td>
<td>7.0 (0.5)</td>
<td>12.8 (0.8)</td>
</tr>
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</table>

Mean (SEM) 7.3 (0.4) 11.9 (0.4)

*Mean (SD)
Tab. 3.2. Mean intra-sample ciliary beat frequency (Hz)* calculated by taking measurements from 20 randomly selected cells.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Room temperature</th>
<th>37 °C</th>
</tr>
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<td>6.8 (0.9)</td>
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<td>14.3 (2.4)</td>
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</table>

Mean (SEM) 7.6 (0.2) 12.8 (0.4)

*Mean (SD)
Tab. 3.3. Intra-cell and intra-sample coefficient of variation of the frequency at room temperature.

<table>
<thead>
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Mean 5 18* 0.01 (intra-sample versus intra-cell)
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<th>Intra-sample</th>
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<tr>
<td>Mean</td>
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</table>

* $P < 0.01$ (intra-sample versus intra-cell)
3.5. Discussion

Much of the published literature on the *in vitro* effects of various substances on CBF has suffered from the absence of standardized criteria of investigation. Many reasons can explain the discrepancy of results among workers. The technique for measuring CBF can be one reason, although the methods used at present (photometric techniques, video-recording methods) yield similar absolute values (Greenstone & Cole, 1985). More important can be the experimental temperature, since the ciliary response to pharmacological stimulation differs by varying the temperature of the experimental micro-environment (Clarke & Lopez-Vidriero, 1983). Other factors include interspecies differences (Zahm et al., 1986), the use of freshly collected or cultured cells (Sanderson & Dirksen, 1986), the chemical characteristics of the culture medium and even the size of the explants (Zahm et al., 1986).

The different beating frequencies displayed by each ciliated cell within the same sample of respiratory epithelium and the spontaneous variations in the CBF of the same cell during the experimental period are important factors when analyzing the effects of pharmacological and physiopathological cilio-modulating agents.

The intra-sample (or inter-cell) variability of the CBF is critical when the results on the effect of cilio-active agents are obtained by comparing the mean CBF of different aliquots of cells collected from the same subject. This method was firstly described by Rutland and colleagues (1981) and used to test the effect of lignocaine on the CBF of human
nasal cells. A broad range of values has been reported in the literature regarding the CBF intra-sample variability. Two factors are important to assess the above value: 1) the criteria of cell selection, and 2) the number of cells from which the CBF is measured.

In some studies (Rutland & Cole, 1980, 1981; Yager et al., 1980; Rutland et al., 1983), the CBF has been based only on measurements from cilia noted to be beating vigorously, providing, therefore, higher mean CBF and smaller variation. At the present, most investigators measure the CBF of cells chosen at random, usually by using an eyepiece graticule. This is also done not to ignore cells beating abnormally (Lopez-Vidriero & Clarke, 1982).

The number of cells chosen to represent the mean intra-sample CBF varies among workers. Most of the published results are based on 3 (Konietzko et al., 1981), 10 (Yager et al., 1978; Rutland & Cole, 1980; Dulfano et al., 1981; Rutland et al., 1982b; Low et al., 1984; Smallman et al., 1984; Wilson et al., 1986; Stanley et al., 1986; Han et al., 1990), 15 (Rossman et al., 1984), or 20 (Greenstone et al., 1988) measurements. It is difficult to compare the above results, since the individual CBF variation is rarely reported. In our study we took 20 measurements from each sample, obtaining mean CoV of 18% at room temperature and 17% at 37°C. If we consider only the CBF of the first 10 cells randomly selected for each sample, we obtain similar mean CoV (18% at room temperature and 20% at 37°C; P > 0.05). In a recent study Teichtahl and colleagues (1986) suggested that the minimum number of epithelial areas
to be examined from one specimen is ten.

When a perfusion chamber preparation is used, the experimental procedure for controlling the effect of cilio-active agents consists of immersing a piece of mucociliary epithelial explant in tissue culture medium and then recording the variations of CBF after the agent under investigation has been added to the culture medium. The results of such an experiment depend on the actual effect of the perfused agent, but also on the spontaneous variations of the CBF during the experimental period. No study has been published on the intra-cell time-related fluctuations of the CBF of human respiratory epithelium under continuous perfusion with culture medium.

As Kennedy and Duckett (1981) emphasised, there is not a unique and constant frequency for all ciliated cells even within a small part of the respiratory epithelium. By using Fast Fourier Transform analysis, the authors observed the existence of fluctuations in individual rabbit tracheal epithelial cells. These results have been confirmed in the frog palate (Eshel et al., 1985), where it has been demonstrated that the frequency variations are not artifacts of the measuring or analyzing system, but are an intrinsic property of ciliary beating (at least in vitro): the frequency of a cilium or group of cilia is time dependent and changes over relatively short periods of time. Zahm and colleagues (1986) studied frog palate and rat tracheal cells under continuous perfusion for 2 hours. The mean intra-cell CoV of the frequency was 19.1% for the frogs (at 26°C) and 10.6% for the rats (at 37°C). The lower CoV found in our study
(4% at 37°C) could be due to interspecies differences and/or the shorter period of observation.

Despite the discrepancies of results on the effect of cilio-active agents on CBF, there is a general agreement in the literature on the basal CBF of human respiratory ciliated cells in vitro. Nasal and bronchial cilia beat at about 13 Hz at 37°C, but considerably more slowly (about 6 Hz) at room temperature (Greenstone & Cole, 1985). There is a positive correlation between CBF in human nasal and bronchial cilia, implying that in health the beating of nasal cilia is indicative of beat frequency lower down the respiratory tract. Within the lower respiratory tract, however, there is a gradient of beat frequencies between subsegmental airways and trachea, with an increase in CBF in the more central airways (Rutland et al., 1982b). Our results confirm the generally accepted values of human nasal CBF in vitro (both at room temperature and at 37°C).

We conclude that the intra-cell and intra-sample variability of the CBF is an important factor when assessing the effects of cilio-active agents on ciliary activity. Our results can be useful in establishing the significance of results in these studies. The wider question remains as to when a small but statistically significant difference in CBF in vitro also constitutes a physiologically meaningful difference in vivo.
4. THE EFFECT OF PERFUSION ON THE CILIARY ACTIVITY OF
RAT TRACHEA IN VITRO

4.1. Summary

Various types of perfusion chambers are used for in vitro studies on ciliary activity. Little information is given on the flow-rates at which solutions are perfused. It is known that mechanical stimulation of ciliated cells increases their ciliary beat frequency (CBF). In this study we investigated the effect of perfusion on the CBF of rat respiratory epithelium. Culture medium at increasing flow-rates was perfused in a chamber containing rat tracheal rings and the effect on CBF was examined. An increase in CBF was observed over 0.25-1.0 ml/min range of flow-rate. After each perfusion experiment the CBF gradually returned to baseline levels in absence of flow. It is concluded that: 1) physical stimulation -perfusion- increases CBF; 2) this finding is important when designing in vitro pharmacological/physiological studies on ciliary activity.

4.2. Introduction

In some of the in vitro methods used for investigating the effects of cilio-active agents a perfusion chamber is used. The main advantages of using a perfusion chamber are that measurements of ciliary activity can be taken from the same group of cilia over long periods of time and that
increasing concentrations of the agent or different agents can be perfused continuously. This is important because of the lower spontaneous variation in ciliary beat frequency (CBF) in the same ciliated cell than in different cells of the same sample (Dulfano et al., 1981; Lopez-Vidriero & Clarke, 1982; Chapter 3.).

In some studies where a perfusion chamber has been used, little information is given regarding the flow-rates at which the solutions were perfused (Verdugo, 1980; Ohashi et al., 1983). There is evidence that mechanical stimulation of ciliated cells increases their CBF (Murakami & Eckert, 1972; Sanderson & Dirksen, 1986) and the rise occurs not only by dimpling on the cell membrane, but also by turbulence of the bath solution. In this study we investigated the effect of perfusion, as a mechanical stimulus, on the CBF of rat respiratory epithelium in vitro. This is important for designing the experimental conditions for pharmacological/physiological studies using perfusion chamber preparations.

4.3. Materials and methods

Sample collection. Since perfusion at high flow-rates could affect the position of the samples in the chamber and, therefore, ampere the measurement of CBF from the same ciliated area throughout the experiment, we used rat tracheal rings. The appropriate thickness of the explants was chosen (see page 49) which prevented movements of the sample during perfusion, while still allowing free motility of the cilia.
Twelve Sprague-Dawley rats weighing between 100 and 120 g were killed by a blow to the head. Tracheal rings were obtained according to the technique described at page 49. Only one intact ring with a heavily ciliated surface was studied per trachea. Each ring was placed in a tissue culture chamber (see page 52) and equilibrated in culture medium (Medium 199, Flow Laboratories) at room temperature for 30 minutes before the study.

**Experimental protocol.** CBF was measured at 21.0 ± 1.3°C. Baseline CBF measurements (B) were taken from one group of cilia on each ring and the same group was observed throughout the whole experiment. Each CBF value represents the mean of 5 consecutive readings of acceptable quality. Fresh culture medium was then perfused at increasing flow-rates (0.1, 0.25, 0.5, 0.75, 1.0 ml/min) for the time required to wash the chamber with 2.5 ml of solution. For each flow-rate, measurements were taken at different times: half-time during perfusion (P), and 1, 5, 10, 15 minutes after the perfusion was stopped (T₁, T₅, T₁₀, T₁₅).

**Statistical analysis.** The results were expressed as means ± SEM. Statistical differences between CBF values were determined by a non parametric Wilcoxon test. Significance was accepted when \( P < 0.05 \).

**4.4. Results**

The experimental results are summarized in Fig. 4.1. (A, B, C, D, E). Since no significant differences emerged between baseline and T₁₅ values at different flow-rates, each T₁₅ value was taken as the baseline for the following flow-rate experiment.
Legend at page 85
Legend at page 85
Fig. 4.1. The effects of increasing rates of perfusion (0.01 -A-; 0.25 -B-; 0.5 -C-; 0.75 -D-; 1.0 ml/min -E-) on ciliary beat frequency (CBF).

CBF values as mean ± SEM.

B = baseline; P = measurements during perfusion; T1, T5, T10, T15 = measurements 1, 5, 10, 15 minutes after perfusion stop.

* P < 0.05, ** P < 0.01 (CBF values versus baseline).
During perfusion at all flow-rates, except 0.1 ml/min, the CBF was significantly increased from baseline. The increase in CBF was flow-rate dependent, the lowest increase was 18% at 0.25 ml/min and the highest 40% at 1.0 ml/min.

When the perfusion was discontinued the CBF gradually returned to baseline values. For the flow-rate 0.25 ml/min CBF had returned to baseline after one minute of stopping the perfusion. At higher flow-rates the effect of perfusion was longer and for 0.75 ml/min CBF was still significantly higher at T₅. For 0.5 ml/min and 1.0 ml/min CBF was significantly higher at T₁₀ and by T₁₅ all values had returned to pre-perfusion values. The spontaneous variation of the frequency calculated at B and T₁₅ during all the experimental period was 12.4 ± 1.3% (mean ± SD).

4.5. Discussion

Ciliary activity can be adjusted in response to changes of the environmental conditions. In non-mammalian ciliated systems, environmental information is frequently transduced into changes in ciliary activity by Ca²⁺-dependent signalling. In Paramecium and Styloynchia, physical stimulation of the anterior cell surface, usually arising from collision during normal movement, results in the reversal of the ciliary beat pattern and a concurrent increase in beat frequency (Naitoh & Eckert, 1974). Similarly, mechanical stimulation has been found to increase, but not reverse, the beat frequency in salamander
oviduct (Murakami & Eckert, 1972), frog palate (Aiello, 1974) and rabbit tracheal (Sanderson & Dirksen, 1986) cilia.

This study is in agreement with previous observations that mechanical/physical in vitro stimulation of ciliated epithelium results in an increase in CBF. It is known that activation (increase in frequency of beating) occurs when the ciliated cells are mechanically stimulated by turbulence of the bath solution (Murakami & Eckert, 1972), thus changes in tone or conformation of the cell membrane caused by liquid flowing over the cell surface can affect the CBF. Our results showed that the CBF response depended on the intensity of the applied stimuli, since higher flow-rates caused higher increases in CBF. This is in agreement with the study of Murakami and Eckert (1972) on salamander oviduct, where they stimulated the ciliated cells with a glass microstylus and found that the peak frequency varied with stylus displacement. In contrast, Sanderson and Dirksen (1986) reported that cultured ciliated cells derived from rabbit tracheal mucosa responded with similar increases in CBF despite variations of the intensity of the stimulus; our study, however, confirms their observation that the duration of the response increases following stronger stimulation.

There is evidence that mechanosensitivity of ciliated cells is dependent on extracellular Ca$^{2+}$ that moves across the cell membrane by means of calcium channels. Both in salamander oviduct (Murakami & Eckert, 1972) and rabbit tracheal cells (Sanderson & Dirksen, 1986) the ability of the cells to respond to mechanical stimulation was lost when
extracellular Ca\textsuperscript{2+} was removed. In the salamander oviduct the increase in CBF was greatly reduced in the presence of lanthanum, which is known to block Ca\textsuperscript{2+} influx across certain cell membranes that normally undergo increased calcium conductance during excitation (Hagiwara & Takahashi, 1967; Miledi, 1971). Similarly, the frequency increase of rabbit tracheal cells was blocked by verapamil, a calcium channel blocker (Lee & Tsien, 1983), in the presence of Ca\textsuperscript{2+} (Sanderson & Dirksen, 1986). In the same study the authors reported that the mechanosensitivity response was similar at room and body temperatures.

A wide range of flow-rates has been used by different authors: 0.02 ml/min (Wanner et al., 1983; Pedersen, 1983; Abraham et al., 1986; Maurer & Liebman, 1988), 0.15 ml/min (Roth et al., 1985; Lopez-Vidriero et al., 1985), 0.2 ml/min (Stafanger, 1987), 0.7-1.0 ml/min (Eckert & Murakami, 1972), 1.0 ml/min (Yanaura et al., 1981b), 2.0 ml/min (Verdugo et al., 1980) and in some studies no information is given (Verdugo, 1980; Ohashi et al., 1983).

We found that, \textit{in vitro}, the rate of perfusion affected CBF and this could influence the assessment of cilio-effects of drugs or chemical mediators when similar preparations are used. The time required after perfusion stop to reverse this effect on CBF was related to the intensity of the flow-rate. Thus, it is important to choose the appropriate flow-rate for each study design, in order to avoid the perfusion influence, particularly when the CBF measurements have to be taken as soon as possible, not to miss rapid modifications in CBF due to the substance
under investigation. We observed that a flow-rate of 0.1 ml/min did not influence CBF. However, the use of low flow-rates affects the possibility of assessing dose-response curves, because of the long time required to reach the stated concentration in the chamber. In order to balance these factors, in our pharmacological studies involving this technique we prefer to perfuse at a flow-rate of 0.25 ml/min with 1 minute interval between the stop of the perfusion and the measurement of CBF.
5. THE EFFECT OF CRYOPRESERVATION ON CILIARY BEAT FREQUENCY OF HUMAN RESPIRATORY EPITHELIUM

5.1. Summary

The effect of cryopreservation on ciliary beat frequency (CBF) of human nasal respiratory epithelium was evaluated. Samples were cryopreserved in a solution containing nutrient medium, 10% foetal calf serum and two different concentrations (10% or 20%) of dimethyl sulfoxide (DMSO) and stored in liquid nitrogen at -196°C for two weeks. CBF of the samples before and after cryopreservation were compared. Mean CBF values before and after cryopreservation did not differ significantly with both concentrations of DMSO. The mean intra-sample coefficient of variation of the CBF decreased significantly after cryopreservation \( (P < 0.05) \). After thawing, CBF remained unchanged for at least 4 hours. It is concluded that normal ciliated epithelial cells can be frozen and stored in liquid nitrogen at -196°C while maintaining their basal CBF.

5.2. Introduction

The increasing interest in investigating ciliary structure and function led to the need to collect great numbers of mucosal biopsies from patients and controls. The limited number of laboratories with facilities to study ciliary activity reduces the quantity and the quality of samples.
available for investigation, since the biopsies must be collected adjacent to where the technique is being used. The difficulty of storing samples for long periods of time leads to the necessity to collect fresh explants every time from the same or from different subjects.

Although respiratory ciliated epithelium can be cultured over long periods of time without losing its mucus production and ion/water transport activity (Verdugo, 1984), there is evidence that the epithelium differentiates into a non-ciliated type and, therefore, is not suitable for studying ciliary activity and structure.

Freezing and storing at very low temperatures have been previously used for preserving human respiratory epithelium (Morris et al., 1973; Wulffraat et al., 1985), although with different techniques and with different results concerning the minimal concentration of protective additive, namely dimethyl sulphoxide (DMSO), needed for a satisfactory recovery of ciliary activity. These studies have shown that cryopreservation does not affect ciliary activity, but they used qualitative or semi-quantitative evaluations (no, slow, or fast ciliary movement).

In this study we investigated the effect of cryopreservation on normal human respiratory epithelium using an accurate quantitative technique for measuring ciliary beat frequency (CBF).

5.3. Materials and methods

Sample collection. The material for this study was obtained from 10 healthy volunteers, ages 23 to 29 years (mean 27.1 years). This group
included eight men and two women; four of them were smokers. None of them reported previous history of upper respiratory tract infections for at least 1 month. Samples of ciliated epithelium were obtained with the nasal brushing technique (see page 49). Adherent epithelium was dislodged from the brush by brisk agitation in a 5 ml plastic bijoux containing 1 ml of culture medium (Medium 199, Flow Laboratories). Some of the strips of epithelium, easily seen in the vial, were immediately transferred with a Pasteur pipette to a sealed coverslip microscope slide preparation for CBF measurements (see page 52). After examination, each sample was transferred back to the same vial for freezing. The same type of microscope preparation was used for CBF measurements of the samples thawed after cryopreservation.

**Measurements of CBF.** Measurements of CBF were carried out at 37°C. The preparations were allowed to equilibrate on the microscope stage for 30 minutes, in order to recover from any trauma that might have occurred during the collection and manipulation procedures. Each CBF value represents the mean of measurements in six different ciliated clumps of the sample, with five readings of acceptable quality on each clump. The choice of the ciliated areas under investigation was randomised (see page 55) and was not limited to the cilia beating vigorously; this was done not to ignore groups of cilia beating abnormally (Lopez-Vidriero & Clarke, 1982).

**Freezing technique.** Each 1 ml suspension was divided into two aliquots of 0.5 ml. Different concentrations of DMSO were added to each
aliquot and then they were frozen following a previous reported technique (Wulffraat et al., 1985). Each aliquot was transferred to a 2 ml plastic ampoule and a mixture of Medium 199, foetal calf serum (FCS) and DMSO was added to reach final concentrations of DMSO of 10% and 20%, respectively. The concentration of FCS was similar in both aliquots (10%). The ampoule was then cooled to 0°C in ice and placed in an isolating polystyrene box (10 x 10 x 6.5 cm; thickness, 1.5 cm). The box was kept in a freezer (Fisons Cliffco) at -70°C for 24 hours, after which the ampoule was transferred to a container with liquid nitrogen (-196°C) and stored for two weeks. Rapid thawing was carried out in a water bath at 37°C, the ampoule being shaken until melting was complete; the solution was then diluted with an equal volume of a mixture of Medium 199 (90%) and FCS (10%) and the sample was examined for CBF measurements. In this set of experiments the observer was unaware of the origin of the sample: fresh samples collected in the same day and cryopreserved samples were coded and studied at random.

Statistical analysis. CBF values were expressed as means ± SD. Statistical differences between means were determined by a paired Wilcoxon test. Significance was accepted when $P < 0.05$.

5.4. Results

Brushings yield relatively small specimens and the ciliated cells are mainly seen as small clumps. It is therefore expected that some will be lost during sampling and multiple transfers. Consequently it was not
possible to determine the exact cell loss due to cell lysis during cooling and thawing. However, in all cases it was possible to find enough cells with beating cilia to take CBF measurements on at least six clumps. In two samples frozen with 10% DMSO and in three samples frozen with 20% DMSO it was necessary to study two slide preparations to perform the measurements.

CBF values are shown in Tables 5.1. and 5.2.. A small and statistically non significant decrease in CBF was observed after cryopreservation in the 10% DMSO samples (-4%) and in the 20% DMSO samples (-9%). No significant difference was found when the two procedures were compared.

Before cryopreservation the mean intra-sample coefficient of variation (CoV) of the frequency was 16%; it decreased to 12% for the samples frozen with 10% DMSO (Fig. 5.1.) and to 11% for those frozen with 20% DMSO (Fig. 5.2.); both decreases were statistically significant (\( P < 0.05 \)).

Samples after cryopreservation still had beating cilia with no variations of the frequency after at least four hours of observation.

5.5. Discussion

The possibility of freezing and storing samples of respiratory epithelium while maintaining ciliary motility has been claimed by previous studies. Morris and colleagues (1973) described a cryopreservation technique to store human embryonic trachea, where recovery of ciliary activity was estimated only by visual observation.
Tab. 5.1. Ciliary Beat Frequency (Hz)*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Before cryopreservation</th>
<th>After cryopreservationb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.7 (1.8)</td>
<td>11.6 (1.1)</td>
</tr>
<tr>
<td>2</td>
<td>11.2 (2.3)</td>
<td>10.2 (1.5)</td>
</tr>
<tr>
<td>3</td>
<td>13.0 (1.8)</td>
<td>13.2 (0.5)</td>
</tr>
<tr>
<td>4</td>
<td>12.7 (2.9)</td>
<td>9.9 (1.9)</td>
</tr>
<tr>
<td>5</td>
<td>10.0 (1.3)</td>
<td>12.4 (1.5)</td>
</tr>
<tr>
<td>6</td>
<td>12.8 (2.9)</td>
<td>11.5 (1.5)</td>
</tr>
<tr>
<td>7</td>
<td>10.3 (1.3)</td>
<td>10.1 (1.6)</td>
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<tr>
<td>8</td>
<td>12.1 (2.3)</td>
<td>11.2 (1.5)</td>
</tr>
<tr>
<td>9</td>
<td>10.9 (0.7)</td>
<td>10.8 (1.1)</td>
</tr>
<tr>
<td>10</td>
<td>11.4 (1.7)</td>
<td>11.2 (0.6)</td>
</tr>
</tbody>
</table>

Mean (SEM) 11.7 (0.3) 11.2 (0.3)^NS

*Mean (SD) intra-sample frequency

^b10% dimethyl sulphoxide

^NSNot significant versus values before cryopreservation
Tab. 5.2. Ciliary Beat Frequency (Hz)*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Before cryopreservation</th>
<th>After cryopreservation&lt;sup&gt;b&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>12.7 (1.8)</td>
<td>9.4 (0.5)</td>
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<tr>
<td>2</td>
<td>11.2 (2.3)</td>
<td>10.7 (1.8)</td>
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<tr>
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<td>13.0 (1.8)</td>
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<td>12.7 (2.9)</td>
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<td>12.1 (2.3)</td>
<td>11.2 (0.8)</td>
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<td>9</td>
<td>10.9 (0.7)</td>
<td>10.9 (1.2)</td>
</tr>
<tr>
<td>10</td>
<td>11.4 (1.7)</td>
<td>11.6 (1.3)</td>
</tr>
</tbody>
</table>

Mean (SEM) 11.7 (0.3) 10.7 (0.4)<sup>NS</sup>

<sup>*Mean (SD) intra-sample frequency</sup>

<sup><sup>b</sup>20% dimethyl sulphoxide</sup>

<sup><sup>NS</sup>Not significant versus values before cryopreservation</sup>
Fig. 5.1. Coefficient of variation (CoV) of the ciliary beat frequency before and after cryopreservation with 10% dimethyl sulphoxide.

* $P < 0.05$. 
Fig. 5.2. Coefficient of variation (CoV) of the ciliary beat frequency before and after cryopreservation with 20% dimethyl sulphoxide.

* $P < 0.05$. 

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Wulfraat and co-workers (1985) reported a freezing technique for nasal mucosal biopsies and observed, with a video-recording technique, that all the frequencies and coordination patterns seen before cryopreservation could be found after cryopreservation. Ciliary activity was described as absent, slow, or fast using a semi-quantitative method. Also, there is experience in the cryopreservation of ciliated protozoans (Simione & Daggett, 1976). In 1976, Shepard and colleagues reported a freezing technique for human renal epithelial carcinoma cells. They showed that slow freezing rates and rapid thawing rates provide the highest post-freeze viability.

It is well known that the survival of cells after freezing depends on three major variables: cooling rate, type and concentration of protective additives, and warming rate (Mazur, 1984).

Morris and colleagues (1973) showed that maximal survival of human embryonic trachea, as judged by ciliary activity and growth of influenza virus, occurred with a slow cooling rate (0.3°C min⁻¹). In order to use a simple technique of cryopreservation, we did not control the exact cooling rate, although the method employed a slow cooling rate at least in what Mazur calls the "lethal intermediate zone of temperature (-15 to -60°C)" (Mazur, 1984).

Regarding the type and concentration of protective additive, we used DMSO, according to previous studies on respiratory epithelial cells (Morris et al., 1973; Wulfraat et al., 1985). We used the minimal concentration of DMSO required according to Wulfraat and colleagues.
(1985), namely 10%, although a previous study (Morris et al., 1973) showed that only when DMSO concentrations of 20% or more were used was there recovery of ciliary activity; however, the difference between the procedures must be considered. We also added 10% of FCS according to Morris and colleagues (1973), who demonstrated no recovery in ciliary activity when serum was omitted.

Previous workers (Morris et al., 1973; Wulffraat et al., 1985) have shown that rapid thawing rates provide the highest post-freeze viability of ciliated respiratory cells and therefore we followed the same procedure.

In our investigation CBF values before and after cryopreservation did not differ significantly and, although the lack of an acceptable level of statistical difference could be due to a type II error (false negative) (Altman, 1980, 1983), mean differences of -4% and -9% in CBF with both concentrations of DMSO are largely in the range of the spontaneous variation of the frequency in fresh samples (+ 16%). The CBF after cryopreservation did not change significantly on increasing the cryoprotective additive concentration, suggesting that a concentration of DMSO of 10% is sufficient for a satisfactory protection against the damages on the cell structures due to freezing and thawing procedures, at least when the above cryopreservation technique is utilized.

The mean intra-sample CoV of the frequency after cryopreservation was significantly lower than that in the fresh samples.

These findings -unchanged mean CBF and reduction in mean intra-sample CoV of the frequency- could be due to the loss of cells with
slower beating cilia and the decrease in frequency of the faster beating cilia. Cells with slow-beating cilia are likely to be abnormal and/or damaged during the sampling manoeuvres and therefore more vulnerable during freezing and thawing procedures.

Our finding that the cilia after cryopreservation continued to beat at the same frequency for at least four hours confirms the similarity of behaviour between samples before and after storage in liquid nitrogen (Dulfano et al., 1981).

In our study the storage time was two weeks. A shorter time (6 days) was used by Wulffraat and colleagues (1985). Since storage temperatures below -130°C effectively stop biological time (Mazur, 1984), we can assume that samples can be stored for indefinite periods of time.

It is concluded that ciliated epithelial cells, at least from healthy subjects, can be stored in liquid nitrogen while maintaining their CBF. Further studies are needed to investigate the possibility of also applying this technique in epithelium from patients with respiratory tract disorders, particularly when modifications of cell membrane properties are involved, as a cause or as a consequence of disease.
6.1. Summary

The effect of terbutaline sulphate on the ciliary activity of fresh and cryopreserved human nasal epithelium was evaluated. Cryopreservation had no effect on baseline ciliary beat frequency (CBF). Both fresh and cryopreserved samples exposed to $10^{-4}$ M terbutaline showed statistically significant ($P < 0.01$) higher CBF values (+27% and +25% respectively) than control. When the percentage differences after drug challenge for fresh and cryopreserved samples were compared no statistical difference emerged. It is concluded that cryopreservation in liquid nitrogen at -196°C does not affect the ciliary response to membrane receptor stimulation, at least beta adrenergic receptors, and therefore cryopreserved samples are suitable for pharmacological studies on ciliary activity.

6.2. Introduction

There is increasing interest in investigating the effects of chemical mediators and pharmacological agents on the ciliary activity of human respiratory epithelium. The direct action of pharmacological, chemical and physical agents on the intrinsic activity of the ciliated cells cannot be investigated using preparations in which mucous secretion is present.
(Wanner, 1977), because of the complex nature of the mucociliary interface. It has been suggested that the interaction of the cilia and mucus might lead to the mechanical stimulation of the cell and to secondary elevation of the ciliary beat frequency (CBF) (Sanderson & Dirksen, 1989). In order to separate the effects of mucus induced stimulation of CBF, in vitro preparations have to be used, where mucus is absent or, if present, very diluted. These methods require a relatively large number of biopsies from normal subjects and patients.

The availability of samples together with the limited number of laboratories with facilities for studying ciliary activity, makes this type of study difficult. However, the possibility of storing samples of ciliated epithelium for long periods of time allows them to be sent from one laboratory to another and to study samples collected in the same occasion at different intervals of time, or, simply, at a more suitable moment.

It has been shown that human respiratory epithelium can be easily stored in liquid nitrogen while maintaining its activity ciliary (Morris et al., 1973; Wulffraat et al., 1985) and we have demonstrated that human respiratory ciliated cells maintain their baseline CBF after cryopreservation for a period of two weeks (Chapter 5.). It is still unknown if this storage procedure affects other aspects of ciliary motility, such as direction of ciliary beat, synchronization, coordination and/or response to physical, chemical or pharmacological agents able to modify ciliary activity.

We, therefore, investigated the effects of terbutaline sulphate, a beta,

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agonist which is known to enhance human CBF in vitro (Clarke & Lopez-Vidriero, 1983) and mucociliary clearance in vivo (Santa Cruz et al., 1974), on the CBF of freshly collected human nasal epithelium and after cryopreservation in liquid nitrogen at -196°C for two weeks.

6.3. Materials and methods

Sample collection. The material for this study was obtained from 10 normal subjects, 5 men and 5 women (mean age 27.3 years, range 23-29; non smokers 7, smokers 3). Samples of ciliated epithelium were obtained, without local anaesthetics, from the inferior nasal turbinate using a nylon brush (see page 49). Adherent epithelium was dislodged from the brush by brisk agitation in a 5 ml plastic bijoux bottle containing 1 ml of culture medium (Medium 199, Flow Laboratories).

Experimental protocol. Each sample was divided into two aliquots. One aliquot was used to study the effects of terbutaline sulphate on the CBF of freshly collected cells. Cells were transferred to coded vials containing either terbutaline sulphate dissolved in Medium 199 or Medium 199 alone. The final drug concentration was $10^{-4}$ M, which is known to produce a maximal stimulatory effect on CBF (Van As, 1974); the pH was adjusted to 7.23. The coded vials were selected at random and aliquots of cells were transferred to sealed coverslip microscope slide preparations (see page 52). CBF measurements were carried out at room temperature ($21.4 \pm 0.7^\circ$C). In order to recover from any trauma that might have occurred during the manipulation procedures, the
preparations were allowed to equilibrate over 15 minutes on the microscope stage. Each CBF value represents the mean of measurements taken in six different ciliated clumps of the sample chosen at random; five readings of acceptable quality were taken on each clump. The choice of the ciliated areas under investigation was randomized (see page 55).

*Freezing technique.* The remaining aliquot of the sample was frozen in liquid nitrogen (Chapter 5.) for two weeks. In this study the foetal calf serum concentration was 10%. After thawing, CBF measurement at baseline and after drug challenge were performed following the same procedure utilized for the fresh samples.

*Statistical analysis.* CBF values were expressed as mean ± SD and the responses as percent changes from the baseline CBF. Statistical differences between values were determined by a paired Wilcoxon test. Significance was accepted when $P < 0.05$.

### 6.4. Results

The CBF for fresh samples of human nasal ciliated epithelium exposed *in vitro* to $10^{-4} \text{M}$ terbutaline sulphate are shown in Figure 6.1.. CBF was significantly higher ($P < 0.01$) than baseline, with a mean percentage difference of +27%.

Cryopreservation had no statistically significant effect on baseline CBF (Tab. 6.1.), with a mean decrease of only 2%. CBF of cryopreserved samples exposed to the drug showed significant higher CBF ($P < 0.01$) than baseline, with a mean percentage difference of +25% (Fig. 6.2.).
Fig. 6.1. Ciliary beat frequency (CBF) at baseline (B) and after exposure to $10^4$ M terbutaline (T) of freshly collected samples.

* $P < 0.01$. 
## Tab. 6.1. Ciliary Beat Frequency (Hz)*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Before cryopreservation</th>
<th>After cryopreservation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.7 (1.1)</td>
<td>6.0 (0.5)</td>
</tr>
<tr>
<td>2</td>
<td>6.5 (0.5)</td>
<td>6.8 (0.7)</td>
</tr>
<tr>
<td>3</td>
<td>6.4 (1.1)</td>
<td>6.6 (1.1)</td>
</tr>
<tr>
<td>4</td>
<td>7.7 (0.9)</td>
<td>6.4 (0.8)</td>
</tr>
<tr>
<td>5</td>
<td>6.6 (0.8)</td>
<td>6.2 (0.7)</td>
</tr>
<tr>
<td>6</td>
<td>6.4 (0.3)</td>
<td>6.7 (0.7)</td>
</tr>
<tr>
<td>7</td>
<td>7.6 (1.0)</td>
<td>7.4 (0.2)</td>
</tr>
<tr>
<td>8</td>
<td>6.0 (0.6)</td>
<td>6.0 (0.5)</td>
</tr>
<tr>
<td>9</td>
<td>6.8 (0.3)</td>
<td>7.4 (0.6)</td>
</tr>
<tr>
<td>10</td>
<td>5.6 (0.3)</td>
<td>5.4 (0.9)</td>
</tr>
</tbody>
</table>

Mean (SEM) 6.6 (0.2) 6.5 (0.2)*NS

*Mean (SD) intra-sample frequency

NS Not significant versus values before cryopreservation

107
Fig. 6.2. Ciliary beat frequency (CBF) at baseline (B) and after exposure to $10^{-4}$ M terbutaline (T) of cryopreserved samples.

* $P < 0.01$. 
When the percentage differences after drug challenge for fresh and cryopreserved samples were compared no statistical difference emerged.

6.5. Discussion

The effect of pharmacological agents on the intrinsic activity of ciliated cells cannot be investigated using preparations in which mucous secretion is present, since changes in the so called 'mucociliary wave' can reflect changes in both mucus and ciliary activity as well as in the periciliary layer. Regulation of ciliary activity can only be investigated using \textit{in vitro} preparations where mucus is absent or, if present, very diluted (Wanner, 1977).

The control of ciliary activity has been studied mainly in respiratory epithelium from different animal species and, although ciliary structure is very similar throughout the phylogenetic series, ciliary function and, in particular, mechanisms of control are not uniform (Sleigh, 1977). The use of human tissue for studying the effects of therapeutic pharmacological agents is preferable and freshly obtained ciliated epithelium is likely to have more physiological relevance than cultured tissue (Rutland & Cole, 1980).

Pharmacological studies require a large number of samples and the availability of fresh explants is critical for these investigations. In addition they are often time consuming and only a limited number of experiments can be carried out in a day. Storage of tissue using methods that do not influence or modify its response, i.e. number and sensitivity
of receptors, will allow the investigators to perform the studies when convenient.

Recently, storage in liquid nitrogen at -196°C has been shown to be an easy and effective way to preserve ciliated cells for relatively long periods of time. Previous studies (Morris et al., 1973; Wulffraat et al., 1985) have demonstrated that it is possible to obtain a satisfactory recovery of ciliary activity after freezing and we have shown that the samples maintain their basal CBF (Chapter 5.).

However, the fact that spontaneous ciliary activity is maintained at the same frequencies does not necessarily imply that the regulatory mechanisms are not affected by this freezing procedure, particularly the response to drugs acting on membrane receptors. This is especially important since the cell membrane is the main target for damage occurring during freezing and thawing (Mazur, 1984).

Our results confirm previous studies on the stimulatory effect of adrenergic agonists on human respiratory cilia: $10^{-6}$ g/ml of reproterol caused a mean increase in human bronchial CBF of 16% (Hesse et al., 1981) and $10^{-4}$ M terbutaline was found to stimulate CBF in bronchial biopsies by 40% (Clarke & Lopez-Vidriero, 1983) at room temperature. At 37°C the effect of terbutaline was less marked (Clarke & Lopez-Vidriero, 1983) and in another study, using nasal epithelium, isoprenaline had no effect (Greenstone & Cole, 1983). We, therefore, performed the study at room temperature, in order to have wider ranges of increase to compare. Our results showed a mean increase of 27% with $10^{-4}$ M
terbutaline, a value within the range found by other workers (Hesse et al., 1981; Clarke & Lopez-Vidriero, 1983).

Our study confirms that cryopreservation has no effect on basal CBF (Chapter 5.) and that the ciliary response to membrane receptor stimulation, at least beta adrenergic receptors, is not modified or reduced, since the response to terbutaline was similar to that observed in fresh samples. Further studies are needed to confirm the similarity of behaviour between fresh and cryopreserved samples in other aspects of ciliary activity.
7. THE EFFECT OF AMILORIDE ON HUMAN BRONCHIAL CILIARY ACTIVITY IN VITRO

7.1. Summary

Amiloride (AM) reduces mucociliary transport in the frog palate in vitro preparation, whereas in vivo inhalation of AM enhances mucociliary and cough clearance in cystic fibrosis patients. AM influences the bioelectric properties of the respiratory mucosa, and since electrical activity and ciliary activity are strictly related in the beating cell, an effect on ciliary function cannot be excluded. We have, therefore, investigated the effect of AM (10^-7 to 10^-3 M) in vitro on the ciliary activity of ten samples of healthy human bronchial epithelium at 37°C. The baseline ciliary beat frequency (CBF) was 13.7 ± 1.0 Hz (mean ± SD). At concentrations of AM of 10^-4 and 10^-3 M, small statistically significant (P < 0.01) increases in CBF were recorded only after 1 minute of exposure to the drug, with percentage changes of +8.2% and +7.6% respectively. We conclude that: AM increases the ciliary activity of normal respiratory cells, but this does not play a major role in the effect of AM on mucociliary transport.
7.2. Introduction

Mucociliary clearance (MCC) is a composite activity that requires normally beating cilia, a periciliary layer in which the cilia beat, and the presence of material with special rheological properties to act as a mechanical coupler (Lopez-Vidriero, 1981). Changes in the rate of transport can be due to modifications of the ciliary function or of the amount, composition, and physical properties of the overlying mucus or both. From in vivo studies it is impossible to distinguish between these two mechanisms, and selected investigations on each of the components of the mucociliary 'escalator' are needed in order to clarify in which way the agent under investigation affects MCC (Wanner, 1977).

Drugs acting on the transport of ions and water of airway epithelium could influence MCC by their effect on the rheological properties of mucus and/or the volume/depth and composition of the periciliary layer. One of these drugs is Amiloride (AM), which has been studied during the last years in different experimental models. This interest is related to its ability to inhibit sodium reabsorption by certain epithelia (Benos, 1982). There is evidence that AM reduces mucociliary transport in vitro in the frog palate preparation (Curtis & Misch, 1984), whereas in vivo inhalation of AM improves the mucociliary and cough clearance in patients with cystic fibrosis (Kohler et al., 1986).

Although it has been assumed that the effect of AM on MCC could be due to modifications in the rheological properties of the secretions, an effect on ciliary activity cannot be excluded. AM influences the bioelectric
properties of the respiratory mucosa (Knowles et al., 1981), and there is a close relationship between electrical activity and ciliary activity in the beating cell (Hakansson & Toremalm, 1966; Yanaura et al., 1979).

We, therefore, investigated the effect of AM on the ciliary activity of human bronchial epithelium in vitro.

7.3. Materials and methods

Sample collection. Samples of bronchial mucosa were obtained during diagnostic fiberoptic bronchoscopy from sites that were macroscopically normal (see page 50). The material was collected from 10 patients, aged 42 to 68 years (mean 55.8 years). The group included 7 men and 3 women; 7 of them were smokers. None of the patients was taking AM, furosemide and/or ouabain at the time of the bronchoscopy. All patients were premedicated with 0.5 mg of atropine i.m., and the airways were anaesthetized with up to 8 ml of 2% lignocaine. Biopsy specimens were taken from the main bronchi (right or left) just proximal to the upper lobe orifice.

Experimental protocol. Each sample was dislodged into a vial containing 5 ml of culture medium (Medium 199, Flow Laboratories) and subsequently transferred to the perfusion tissue culture chamber (see page 52). Each sample was allowed to equilibrate for 30 min on the microscope stage before baseline CBF measurements were taken.

Care was taken to design the perfusion procedure. The chamber was perfused continuously with Medium 199 at a flow-rate of 0.02 ml/min.
Only when the drug (Amiloride hydrochloride, Berck Pharmaceuticals) was added to the solution, the flow-rate was increased to 0.25 ml/min; this was done to reduce the time required to fill the chamber with the concentration of drug under investigation. Again the flow-rate was increased from 0.02 to 0.25 ml/min when the chamber was perfused, after each drug concentration run, with Medium 199 only, to look at the reversibility of eventual effects. This procedure was performed once without drug, acting as a control, and then five times with increasing concentrations of the drug \(10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, \text{and } 10^{-3} \text{M}\).

Ciliary beat frequency (CBF) measurements were carried out at 37°C, the same ciliated area of each sample being observed throughout the experimental period. Measurements were taken at baseline (B), 1 \(T_1\), 15 \(T_2\), 30 \(T_3\) minutes after perfusion with the drug and after washing the chamber with culture medium only \(T_4\) at the end of each concentration run. All measurements were, therefore, taken during perfusion at 0.02 ml/min. Each CBF value represents the mean of ten consecutive readings of acceptable quality.

*Statistical analysis.* The results were expressed as mean \(\pm\) SD. Statistical differences between mean values were determined by a non parametric Wilcoxon test. Significance was accepted when \(P < 0.05\).

7.4. Results

The baseline CBF was 13.7 \(\pm\) 1.0 Hz and the mean intra-sample
coefficient of variation of the frequency was ± 5.2%. After exposure to increasing concentrations of AM between $10^{-7}$ and $10^{-5}$ M (Fig. 7.1., A-B-C), no changes in CBF were recorded. At $10^{-4}$ and $10^{-3}$ M, there were statistically significant ($P < 0.01$) increases only at T1, with percentage changes of +8.2% and +7.6% respectively (Fig. 7.1., D-E).

7.5. Discussion

The increasing interest in investigating the effect of AM on mucociliary transport, mainly related with a possible therapeutic use of the drug in cystic fibrosis patients, addresses a number of issues regarding the basic mechanism of action of the drug on the respiratory epithelium. So far, the effects of the drug have been considered as a consequence of its ability to inhibit active sodium absorption (Benos, 1982) and, therefore, reduce the transepithelial electric potential difference (Knowles et al., 1981) and increase the water content of bronchial mucus (Mentz et al., 1986).

However, its effect on transepithelial potential difference (PD) turns the discussion in terms of bioelectric properties of the respiratory mucosa. Basic studies by Hakansson and Toremalm (1966) have shown that ciliated cells of rabbit trachea have a negative membrane potential with periodically fluctuating components. This oscillating component was considered to function as a 'pace-maker' in order to control ciliary activity. In a study on canine trachea, Yanaura and colleagues (1979) concluded that the electrical activity is closely related to the ciliary
Legend at page 119
Fig. 7.1. The effects of increasing concentrations of Amiloride (10^7 M - A; 10^6 M - B; 10^5 M - C; 10^4 M - D; 10^3 M - E) on ciliary beat frequency (CBF).

CBF values as mean ± SD.

B = baseline; T1, T2, T3 measurements after 1, 15, and 30 minutes of exposure to the drug and after washing with control solution (T4).

* P < 0.01 (CBF values versus baseline).
activity in the beating cell. Consequently, a drug able to modify (Knowles et al., 1981) so dramatically PD (-24% in healthy subjects and -90% in cystic fibrosis patients) could have an effect on ciliary activity as well.

Curtis and Misch (1984) showed that AM inhibited mucociliary transport over the surface of isolated frog palate epithelium and that the rate of ciliary beating was unchanged. However, measurements of ciliary activity were performed using a stroboscopic technique that gives approximate rates of beat frequency and where accurate values are difficult to obtain (Dalhamn, 1970).

In our study, concentrations of AM between $10^{-7}$ and $10^{-5}$ M had no effect on CBF. With higher concentrations ($10^{-4}$ and $10^{-3}$ M) small increases, although statistically significant ($P < 0.01$), were detected, and they were rapid and of short duration, being observed only at $T_1$.

It is unlikely that the increase in CBF observed in our in vitro study on normal cells is responsible for the overall improvement of MCC reported in cystic fibrosis patients (Kohler et al., 1986), since the rise in CBF is small and of very short duration, while the enhancement in MCC in vivo was seen 30 and 60 min after exposure to the drug. On the other hand, the modifications of electric properties caused by AM are of larger magnitude in cystic fibrosis than in normal mucosa (Knowles et al., 1981), and this could be reflected in larger and/or more prolonged increases of CBF.

We conclude that AM increases the activity of normal ciliated cells, but this does not play a major role in the effect of AM on mucociliary
transport.
8. ROLE OF CYCLIC AMP IN REGULATION OF CILIARY BEAT FREQUENCY IN HUMAN RESPIRATORY EPITHELIUM

8.1. Summary

In order to investigate the effects of cyclic AMP on ciliary beat frequency (CBF) in human respiratory epithelium, cells were brushed from the inferior nasal turbinates of three groups of 10 subjects: awake healthy adults, anaesthetized children, and anaesthetized adults. Cells from the awake adults were also studied after 24 hours in tissue culture. CBF was measured in vitro with a photometric technique at room temperature. Samples were mounted in a perfusion chamber and challenged with either control solutions, dibutyryl cyclic AMP (10^-4 or 10^-3 M), or the cyclic nucleotide-dependent protein kinase inhibitor 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H-7). Dibutyryl cyclic AMP (10^-3 M) caused significant increases in CBF in all groups studied: awake adults (+ 1.1 Hz; \( P < 0.01 \)), anaesthetized children (+ 1.2 Hz; \( P < 0.01 \)), anaesthetized adults (+ 1.2 Hz; \( P < 0.01 \)), and cultured cells (+ 1.1 Hz; \( P < 0.01 \)). This response was inhibited by preincubation with H-7 (10^-4 and 10^-3 M). It is concluded that cyclic AMP is a regulator of ciliary activity in human respiratory epithelium.
8.2. Introduction

Neurohormones and neurotransmitters associated with the autonomic nervous system have a significant influence on mucociliary activity (Pavia et al., 1980). It has been demonstrated in vitro that adrenergic drugs cause an increase in ciliary beat frequency (CBF) in both animal (Van As, 1974; Verdugo et al., 1980; Yanaura et al., 1981b; Lopez-Vidriero et al., 1985) and human (Hesse et al., 1981; Clarke & Lopez-Vidriero, 1983; Chapter 6.) tissues. Recent in vivo studies on dogs confirm the role of autonomic agonists in regulating CBF (Wong et al., 1988b). Beta-adrenergic drugs act via membrane receptors and elevate the intracellular concentration of adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Tomlinson et al., 1985). Cyclic AMP acts by binding to intracellular protein receptors and induces the release of the catalytic sub-unit of protein kinase A (PKA), which in turn phosphorylates a range of target proteins (Edelman et al., 1987). We have investigated this pathway in respiratory epithelial cell taken from the nasal turbinates of human subjects.

The aims of this study were: 1) to investigate the regulation of CBF in human respiratory epithelium by exposing the ciliated cells to an analogue of cyclic AMP which crosses cell membranes (dibutyryl cyclic AMP); 2) to block the effect of dibutyryl cyclic AMP on CBF with 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H-7), a competitive antagonist of the adenosine 5'-triphosphate (ATP) binding site of the cyclic AMP-dependent kinase (Hidaka et al., 1984); 3) to determine if the response
to dibutyryl cyclic AMP is affected by the age of the subject, collection of the sample during the induction of general anaesthesia, or storage of the cells in tissue culture for 24 hours.

8.3. Materials and methods

Sample collection. Ciliated nasal epithelium was obtained from the mucosa overlying the nasal turbinates using a cytology brush (see page 49). Two or three brushings were taken from the same nostril of each subject. Cellular material adhering to the brush was dislodged in Eppendorf tubes containing 1 ml of tissue culture medium (Medium 199). All the experiments on fresh samples were performed within 4 hours of collection. During this time the samples were kept in Medium 199 at 4°C.

Subject selection. The material for this study was obtained from three groups of subjects: healthy awake adults, anaesthetized children, and anaesthetized adults.

The awake adults consisted of 10 healthy volunteers, aged 20-34 years (mean 29.2 years). This group included 7 men and 3 women; 4 of them were smokers. None of them had a history of upper respiratory tract infection for at least one month. Brushings from each subject were obtained without using local anaesthesia on two different days. Samples obtained on the first day were divided into two aliquots and used to test the effect of two concentrations of dibutyryl cyclic AMP (10⁻⁴ and 10⁻³ M). Samples obtained on a separate day from the same nostril were placed
in tissue culture and used to investigate the effect of dibutyryl cyclic AMP (10\(^{-3}\) M) after 24 hours.

It was decided not to collect samples from conscious children due to the discomfort of the procedure. We, therefore, obtained Ethical Committee permission to study children who were undergoing routine surgery. This group was composed of 10 children aged 2-15 years (mean 7.3 years; 5 males and 5 females). Samples were collected immediately after induction of general anaesthesia with thiopentone (4 mg/kg) and suxamethonium (1 mg/kg).

In order to control for the effect of general anaesthesia, a group of 10 anaesthetized adults were also studied. These subjects were undergoing routine surgical procedures as above and included 7 men and 3 women, aged 19-61 years (mean 39.7 years).

Signed informed consent was obtained from all subjects, and parental consent was obtained for the children.

*Cell culture.* (see page 51).

*Measurement of CBF.* Ciliated cells were transferred in Medium 199 to a perfusion chamber (see page 52). The chamber was connected to a perfusion pump delivering 0.25 ml/min, which gave a 90% washout of the fluid in the chamber in 10 minutes. 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 (22.0 ± 1.5°C) using the photometric technique (see page 55). In order to eliminate the increase
in CBF induced by perfusion (Chapter 4.) all recordings were taken in the absence of flow. The choice of the cells was randomized (see page 55).

Repeated CBF measurements on a single cell. Following a 30 minute period of equilibration on the microscope stage, the sample in the chamber was perfused for 10 minutes with fresh Medium 199 equilibrated with room air. Baseline values were obtained by taking the mean of CBF measurements after 1, 15 and 30 minutes. Following the baseline readings, the perfusate was replaced with 1) Medium 199 alone or 2) medium supplemented with either butyric acid (2 x 10^{-3} M) or dibutyryl cyclic AMP (10^{4} or 10^{5} M). CBF was measured after 1, 15 and 30 minutes following perfusion. The pH of all solutions was between 7.2 and 7.4, a range known not to affect CBF (Luk & Dulfano, 1983). All the CBF measurement were taken by the same observer, who was unaware of the nature of the perfusates.

Inhibition of the dibutyryl cyclic AMP-dependent effect on CBF. When the protein kinase inhibitor was used, the second perfusate was replaced with either a control solution containing the vehicle (dimethyl sulphoxide, final concentration 1%) or a range of concentrations of H-7 (10^{-5}, 10^{-4}, and 10^{-3} M). When 10^{-5} and 10^{-3} M H-7 were used, the samples were taken from awake adults. In the 10^{-4} M H-7 experiments, the samples were selected at random from all the three previously described groups. CBF measurements were taken every 15 minutes during the 1 hour incubation with H-7 or vehicle. A the end of the hour, the perfusate was replaced
with one containing dibutyryl cyclic AMP ($10^{-3}$ M) and CBF was measured as above (1, 15 and 30 minutes following perfusion).

**CBF measurements on different cells.** The results from the single cell perfusion technique were compared to those from an alternative method which uses a sealed coverslip microscope slide preparation (see page 52). This measures the mean CBF of two separate aliquots of cells from the same brushing, one of which had been exposed to the active agent. Samples from the awake adults were transferred to coded vials containing either $10^{-3}$ M dibutyryl cyclic AMP dissolved in Medium 199 or Medium 199 alone. The pairs of coded vials were selected at random and, after incubation for 10 minutes at room temperature, the aliquots of cells were transferred to sealed microscope preparations for measuring CBF. Twenty consecutive measurements were taken from different ciliated cells in each preparations and the mean CBF was calculated. The areas were randomly chosen by moving the eyepiece graticule across the specimen and the selection criteria for each cell were the same as in the perfusion experiments.

**Effect of phorbol myristic acid on CBF.** H-7 blocks the protein kinase C (PKC) as well as PKA (Hidaka et al., 1984). In order to examine a possible role of PKC in the control of human CBF, we challenged cells from awake adults with phorbol myristic acid (PMA), an activator of PKC, using the sealed microscope preparation. The concentration of PMA used ($10^{-5}$ M) is known to increase protein phosphorylation in these (unpublished data) and other cell types (Schneider et al., 1981).
Chemical reagents. Medium 199 (Flow Laboratories), butyric acid, sodium salt of dibutyryl cyclic AMP, H-7, and PMA (Sigma).

Statistical analysis. CBF values were expressed as the mean ± SEM and the response as the absolute difference from the baseline CBF. Differences in CBF within groups were determined by a paired Wilcoxon test. Differences between groups were determined by a Mann-Whitney U test. Significance was accepted when $P < 0.05$.

8.4. Results

CBF values after perfusion. After perfusion with fresh Medium 199, butyric acid or dibutyryl cyclic AMP there were no significant differences between CBF values recorded at times 1, 15 and 30 minutes. The mean CBF of the three readings was therefore taken to represent the change in CBF with respect to the baseline value due to the substance perfused.

Control experiments. In the awake adult group there was no change in CBF with respect to the baseline when the chamber was perfused with fresh Medium 199, with a mean CBF of $7.8 \pm 0.3$ and $7.8 \pm 0.4$ Hz for baseline and medium respectively (Tab. 8.1.). CBF was also unchanged following perfusion with Medium 199 plus butyric acid ($2 \times 10^{-3}$ M), with means of $7.7 \pm 0.3$ and $7.7 \pm 0.5$ Hz for baseline and butyric acid respectively (Tab. 8.2.). In our preparation the CBF was stable for at least 2 hours, with a mean intra-cell coefficient of variation of the frequency, defined as the ratio of the standard deviation to the mean value as a percentage, of $\pm 8.7\%$ (n = 20).
Tab. 8.1. Effect of Medium 199 on the ciliary beat frequency (Hz) of cells from awake adults.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Baseline</th>
<th>Medium 199</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>7.8</td>
</tr>
<tr>
<td>2</td>
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</tr>
<tr>
<td>10</td>
<td>7.1</td>
<td>7.8</td>
</tr>
</tbody>
</table>

Mean (SEM)  
7.8 (0.3)  7.8 (0.4) <sup>NS</sup>

<sup>NS</sup> Not significant versus baseline values
Tab. 8.2. Effect of butyric acid ($2 \times 10^{-3} \text{ M}$) on the ciliary beat frequency (Hz) of cells from awake adults.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Baseline</th>
<th>butyric acid</th>
</tr>
</thead>
<tbody>
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<td>8.2</td>
</tr>
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</tr>
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<td>7.0</td>
</tr>
<tr>
<td>10</td>
<td>6.8</td>
<td>6.6</td>
</tr>
</tbody>
</table>

Mean (SEM)  
7.7 (0.3) 7.7 (0.5)\textsuperscript{NS}

\textsuperscript{NS}Not significant versus baseline values
Effect of dibutyryl cyclic AMP. Perfusion with Medium 199 plus dibutyryl cyclic AMP (10⁻³ M) caused a statistically significant increase (+ 1.1 Hz; P < 0.01) in CBF in the awake adult group (Fig. 8.1.). The increment in CBF was already present at the time of the first reading following perfusion (1 minute) and did not increase further after 15 and 30 minutes. The increase was sustained after perfusion with fresh Medium 199 for 10 minutes (Fig. 8.1.). CBF did not change following perfusion with the lower concentration of dibutyryl cyclic AMP (10⁻⁴ M), despite continuous observation for 1 hour, with a mean CBF of 7.3 ± 0.3 Hz at baseline and 7.3 ± 0.4 Hz after perfusion with dibutyryl cyclic AMP (Tab. 8.3.). Cells cultured for 24 hours showed the same pattern of response to dibutyryl cyclic AMP (10⁻³ M) as fresh cells (+ 1.1 Hz; P < 0.01) (Fig. 8.2.).

The changes in CBF following perfusion with the higher concentration of dibutyryl cyclic AMP (10⁻³ M) in the anaesthetized children group (+ 1.2 Hz; P < 0.01) (Fig. 8.3.) and the anaesthetized adult group (+ 1.2 Hz; P < 0.01) (Fig. 8.4.) were similar to those described for the awake adults. There were no significant differences in the increments in frequency between groups.

Experiments comparing the mean CBF of different aliquots of cells, using the sealed coverslip microscope slide preparation, confirmed the stimulatory effect of dibutyryl cyclic AMP (10⁻³ M), with a mean increase of 1.0 Hz (P < 0.05) (Tab. 8.4.).

Effect of H-7. Perfusion with H-7 (10⁻⁵, 10⁻⁴, and 10⁻³ M) did not affect
**Fig. 8.1.** Effect of dibutyryl cyclic AMP ($10^3$ M) on the ciliary beat frequency (CBF) of cells from awake adults.

* $P < 0.01$. 
Tab. 8.3. Effect of dibutyryl cyclic AMP ($10^4$ M) on the ciliary beat frequency (Hz) of cells from awake adults.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Baseline</th>
<th>dibutyryl cyclic AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.7</td>
<td>6.0</td>
</tr>
<tr>
<td>2</td>
<td>5.9</td>
<td>5.1</td>
</tr>
<tr>
<td>3</td>
<td>7.0</td>
<td>7.4</td>
</tr>
<tr>
<td>4</td>
<td>6.4</td>
<td>6.0</td>
</tr>
<tr>
<td>5</td>
<td>7.8</td>
<td>7.3</td>
</tr>
<tr>
<td>6</td>
<td>7.8</td>
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<td>7.9</td>
</tr>
<tr>
<td>10</td>
<td>8.1</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Mean(SEM) 7.3 (0.3)  7.3 (0.4)$^{NS}$

$^{NS}$Not significant versus baseline values
Fig. 8.2. Effect of dibutyryl cyclic AMP (10⁻³ M) on the ciliary beat frequency (CBF) of cells from awake adults after 24 hours in tissue culture.

* P < 0.01.
Fig. 8.3. Effect of dibutyryl cyclic AMP (10³ M) on the ciliary beat frequency (CBF) of cells from anaesthetized children.  

* $P < 0.01$. 
**Fig. 8.4.** Effect of dibutyryl cyclic AMP (10^{-3} M) on the ciliary beat frequency (CBF) of cells from anaesthetized adults.

* $P < 0.01$. 
**Table 8.4.** Effect of dibutyryl cyclic AMP ($10^{-3}$ M) on the ciliary beat frequency (Hz) of cells from awake adults (sealed coverslip microscope slide preparation).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Control*</th>
<th>dibutyryl cyclic AMP*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.3 (1.2)</td>
<td>6.0 (1.0)</td>
</tr>
<tr>
<td>2</td>
<td>10.5 (2.1)</td>
<td>12.0 (1.8)</td>
</tr>
<tr>
<td>3</td>
<td>6.6 (0.9)</td>
<td>7.1 (1.3)</td>
</tr>
<tr>
<td>4</td>
<td>6.7 (1.3)</td>
<td>8.2 (1.2)</td>
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<td>5</td>
<td>7.3 (1.0)</td>
<td>9.1 (1.8)</td>
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<td>10.4 (2.8)</td>
</tr>
<tr>
<td>7</td>
<td>8.6 (1.7)</td>
<td>7.5 (0.8)</td>
</tr>
<tr>
<td>8</td>
<td>9.1 (1.1)</td>
<td>10.6 (2.4)</td>
</tr>
<tr>
<td>9</td>
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<td>11.7 (1.7)</td>
</tr>
<tr>
<td>10</td>
<td>9.3 (1.9)</td>
<td>12.0 (1.8)</td>
</tr>
</tbody>
</table>

Mean(SEM) 8.4 (0.5) 9.4 (0.7)*

*Mean (SD) intra-sample frequency

* $P < 0.05$ (versus control values)
the baseline CBF during the 1 hour incubation period. After incubation with $10^{-5} \text{M H-7}$ (Fig. 8.5.), the subsequent exposure to dibutyryl cyclic AMP ($10^{-3} \text{M}$) caused an increase in CBF ($+ 0.8 \text{ Hz}; P < 0.05$). When the cells were incubated for 1 hour with $10^{-4} \text{M}$ (Fig. 8.6.) and $10^{-3} \text{M}$ (Fig. 8.7.) H-7, the expected rise in CBF following perfusion with dibutyryl cyclic AMP ($10^{-3} \text{M}$) did not occur. Preincubation for 1 hour with a control solution containing the vehicle alone did not affect the rise in CBF following exposure to dibutyryl cyclic AMP ($10^{-3} \text{M}$) (Fig. 8.8.).

Effect of PMA. The mean CBF of aliquots of cells from a single brushing were similar, following exposure to PMA ($10^{-5} \text{M}$) or a control solution ($8.6 \pm 0.4$ and $8.5 \pm 0.4 \text{ Hz}$ respectively) (Tab. 8.5.).

8.5. Discussion

Control experiments. In our system the CBF was stable for at least 2 hours, whereas some other groups working with similar perfusion preparations (Maurer & Liebman, 1988; Sanderson & Dirksen, 1989) have noted a decline of CBF during their experiments. In this respect our data resemble the findings of Zahm and colleagues (1986), who recorded a constant CBF for 2 hours during continuous perfusion of rat tracheal explants. It is known that ciliated cells show spontaneous fluctuations in CBF (Kennedy & Duckett, 1981; Chapter 3.) and in our control experiments the mean intra-cell coefficient of variation of the frequency was $\pm 8.7\%$. Perfusion with either fresh Medium 199 or butyric acid did not affect the baseline CBF.
Fig. 8.5. The effect of 1 hour of incubation with $10^{-5}$ M 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H-7) on the ciliary beat frequency (CBF) response to $10^{-3}$ M dibutylryl cyclic AMP. * $P < 0.05$. 
Fig. 8.6. The effect of 1 hour of incubation with $10^4$ M 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H-7) on the ciliary beat frequency (CBF) response to $10^{-3}$ M dibutyryl cyclic AMP.
**Fig. 8.7.** The effect of 1 hour of incubation with $10^{-3}$ M 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H-7) on the ciliary beat frequency (CBF) response to $10^{-3}$ M dibutyryl cyclic AMP.
Fig. 8.8. The effect of 1 hour of incubation with control solution on the ciliary beat frequency (CBF) response to $10^{-3}$ M dibutyryl cyclic AMP.

* $P < 0.01$. 
Tab. 8.5. Effect of $10^{-5}$ M phorbol myristic acid (PMA) on the ciliary beat frequency (Hz) of cells from awake adults.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Control*</th>
<th>PMA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.3 (1.4)</td>
<td>9.3 (1.0)</td>
</tr>
<tr>
<td>2</td>
<td>10.0 (1.5)</td>
<td>9.8 (0.7)</td>
</tr>
<tr>
<td>3</td>
<td>9.1 (1.5)</td>
<td>8.8 (2.2)</td>
</tr>
<tr>
<td>4</td>
<td>7.7 (1.0)</td>
<td>7.5 (0.8)</td>
</tr>
<tr>
<td>5</td>
<td>7.5 (2.4)</td>
<td>8.0 (1.8)</td>
</tr>
<tr>
<td>6</td>
<td>7.7 (1.3)</td>
<td>7.8 (1.5)</td>
</tr>
<tr>
<td>7</td>
<td>7.9 (1.2)</td>
<td>8.2 (1.7)</td>
</tr>
<tr>
<td>8</td>
<td>7.8 (1.0)</td>
<td>7.5 (2.1)</td>
</tr>
<tr>
<td>9</td>
<td>7.5 (1.0)</td>
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</tr>
<tr>
<td>10</td>
<td>10.9 (1.1)</td>
<td>11.2 (0.9)</td>
</tr>
</tbody>
</table>

Mean(SEM)  
8.5 (0.4) 8.6 (0.4)\textsuperscript{NS}

\textsuperscript{*}Mean (SD) intra-sample frequency

\textsuperscript{NS}Not significant versus control values
Pathways controlling CBF. Mammalian respiratory cilia increase their CBF when exposed to sympathomimetic and parasympathomimetic agonists (Sleigh et al., 1988). Animal studies have shown that ciliated respiratory cells have at least two intracellular pathways for the control of CBF: one utilising calcium, the other cyclic AMP (Sanderson & Dirksen, 1989). In an initial attempt to unravel these control mechanisms, we investigated the role of cyclic AMP in the regulation of CBF in human epithelium.

Much evidence has implicated cyclic AMP as the second messenger in many physiological processes since the original report by Sutherland and colleagues (1968). Cyclic AMP has been shown to activate or increase the beat frequency of cilia and flagella (Sanderson & Dirksen, 1989), but most of the studies on the regulation of ciliary activity by cyclic nucleotides have been on unicellular eukaryotes. There have been two reports on the stimulatory effect of cyclic AMP analogues on CBF in canine (Yanaura et al., 1981b) and rabbit (Tamaoki et al., 1989b) tracheal epithelium. There appear to be no published papers on the effect of cyclic AMP on human respiratory tissue, except for one preliminary report on human bronchial cells in tissue culture which showed a maximal increase in CBF of 35.8% with 1 μM 8-bromo-cyclic AMP (Yang et al., 1989).

Effect of dibutyryl cyclic AMP. Dibutyryl cyclic AMP (10^{-3} M) caused a significant increase of CBF. This extracellular concentration of dibutyryl cyclic AMP is similar to that known to increase CBF in rabbit
tracheal epithelium (Tamaoki et al., 1989b) and to open chloride channels in canine respiratory epithelium (Al-Bazzaz, 1981) and in gut mucosa (Peterson & Ochoa, 1989). This concentration of dibutyryl cyclic AMP may at first sight seem to be relatively high. However, Cohn (1987) has found that millimolar concentrations are needed to induce changes in intracellular protein phosphorylation. Furthermore, the altered pattern of phosphorylation was the same as that seen following stimulation of cell surface receptors linked to adenyl cyclase. The explanation for this may lie in the value of the concentration necessary to produce a half-maximal response ($K_D$) for the butyryl analogue with respect to cyclic AMP (Moore et al., 1988). The $K_D$ value for PKA activation for dibutyryl cyclic AMP is $3.5 \times 10^{-5}$ M relative to a value of $3 \times 10^{-8}$ M for cyclic AMP itself. If one assumes that increased activation is seen at 10 times the $K_D$ value, then the reason for the requirement of $10^{-3}$ M becomes clear, when one allows for the concentration gradient across the cell.

Although our experimental design did not allow us to determine the exact time of onset of the CBF response, our data showed that the rise in frequency occurred within 10 minutes of exposure to dibutyryl cyclic AMP ($10^{-3}$ M). Furthermore, the data showed that the effect lasted for at least 30 minutes and was not reversed immediately after washing the chamber with fresh Medium 199. This persistent effect could be due to a long lived change in the phosphorylation state of the target proteins beyond the activation of cyclic AMP-dependent protein kinase. Exposure of cells to $10^{-4}$ M dibutyryl cyclic AMP produced no change in CBF and
there are two possible explanations for this in addition to those discussed above: firstly that it might have been insufficient to overcome the naturally occurring inhibitor of cyclic AMP-dependent protein kinase (Walsh et al., 1971), or secondly that the activity of the intracellular phosphodiesterase might be sufficiently high to degrade the lower dose of dibutyryl cyclic AMP.

The magnitude of the change in CBF following a given stimulus may be affected by a number of factors including interspecies differences (Sleigh, 1977) and, possibly, site of sampling (Bisgaard & Pedersen, 1987). The wide range of CBF responses is illustrated by several examples in the literature using beta-adrenergic agonists in bovine (+250%) (Wong et al., 1988a), rabbit (+100%) (Verdugo et al., 1980), rat (+50%) (Lopez-Vidriero et al., 1985), and human (+18%) (Hesse et al., 1981) ciliated epithelium. Our results showed that CBF increased by an average value of 15% after $10^{-3}$ M dibutyryl cyclic AMP. This result is similar to that seen in rabbit tracheal cells (+19%) using the same analogue of cyclic AMP (Tamaoki et al., 1989b). On a percentage basis, this rise in CBF appears to be small. However, this need not necessarily be taken to imply that such an increment might be physiologically insignificant. An increase of 1.2 Hz, when taken over one hour, results in an extra 4320 beats. This, together with the persistence of the rise even after washing the chamber with fresh culture medium, suggests that the rate of mucus transport might be significantly enhanced following a rise in intracellular cyclic AMP, 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).

When studying the effect of cilio-active drugs and cilio-modulating agents on CBF, the use of freshly obtained ciliated epithelium is preferable. However, these studies are time consuming and the availability of human samples cannot always be predicted. This problem may be overcome in two ways. The samples can either be preserved in liquid nitrogen (Chapter 5.) or placed in tissue culture. It is known that the rise in CBF following mechanical stimulation is dependent on the age of the cells in culture (Sanderson & Dirksen, 1986). In our study, the response to dibutyryl cyclic AMP was similar in fresh cells and in cells cultured for 24 hours in Medium 199 supplemented with 1% foetal calf serum and broad spectrum antibiotics.

Age of subject and effect of anaesthesia. The response of CBF to dibutyryl cyclic AMP was compared in different age groups of subjects. The cells from anaesthetized children responded to dibutyryl cyclic AMP in a similar manner to those from awake adults. Anaesthetics generally decrease ciliary activity in vitro (Iravani & Melville, 1976) and it therefore remained possible that despite our attempt to collect samples from the children immediately after the induction of anaesthesia, the anaesthetic agents might themselves have altered the capacity to respond to cyclic AMP. Since the response of awake adults and those under anaesthetic did not differ, it appears that the in vitro effect of dibutyryl
cyclic AMP on CBF is not affected by the induction of general anaesthesia. This result is supported by the observation of Rutland and colleagues (1981) that local administration of lignocaine prior to brushing did not affect baseline CBF in vitro.

Effect of H-7 on the cyclic AMP-response. H-7 is a complex competitive blocker of the ATP binding site on the catalytic subunit of cyclic nucleotide-dependent protein kinases (Hidaka et al., 1984). H-7 (10^{-4} and 10^{-3} M) blocked the expected rise in CBF following exposure to 10^{-3} M dibutyryl cyclic AMP. 10^{-5} M H-7 failed to prevent the subsequent rise in CBF, but the mean increment in frequency (+ 0.8 Hz) was the lowest we have ever recorded in our studies on dibutyryl cyclic AMP (10^{-3} M). Although this attenuation in response did not reach statistical significance when compared to control experiments, it is likely that H-7 begins to act near this concentration.

Effect of PMA on CBF. H-7 blocks cyclic nucleotide- and calcium phospholipid-dependent protein kinases (Hidaka et al., 1984). We used PMA to stimulate PKC in an initial attempt to exclude a major role for this kinase in the control of CBF. The failure to change CBF with PMA suggests that PKC is not involved in the control of human CBF, whereas there are data supporting an inhibitory role in rabbit cells (Kobayashi et al., 1988).

In conclusion, we have shown that dibutyryl cyclic AMP increases the CBF of respiratory cells from adults and children, and that anaesthesia does not affect this rise. This response is likely to be
mediated by a cyclic AMP-dependent mechanism, because it may be blocked by a competitive antagonist of cyclic nucleotide-dependent kinases.
9. ROLE OF CALCIUM IN REGULATION OF CILIARY BEAT FREQUENCY IN HUMAN RESPIRATORY EPITHELIUM

9.1. Summary

To study the role of calcium in the regulation of human respiratory ciliary activity, the changes in ciliary beat frequency (CBF) of nasal epithelial cells were measured in vitro 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). Addition of $10^{-5}$ M 4-Br-A23187 caused a time-dependent ($P < 0.01$) rise in CBF. The increment in CBF was first detected 10 minutes (+10%; $P < 0.01$) after challenge and was sustained for at least 1 hour, with maximal stimulation after 40 minutes (+18%; $P < 0.01$). Exposure to $10^{-4}$ M 4-Br-A23187 caused an immediate increase in intracellular free calcium concentration, which reached maximal values before the increase in CBF. TFP ($10^{-4}$ M) caused a reduction of baseline CBF (-10%; $P < 0.01$) and prevented the expected rise when the cells were subsequently exposed to 4-Br-A23187. We conclude that: 1) calcium ionophore stimulates the CBF of human respiratory cells; 2) this effect is mediated through a calmodulin-sensitive system; 3) the same pathway
appears to control the basal CBF of these cells.

9.2. Introduction

There is very little information on the second messengers with might regulate ciliary activity in mammalian respiratory cells, but it has been suggested that calcium and cyclic nucleotides might both be important in the control of ciliary beat frequency (CBF) (Sanderson & Dirksen, 1989). Studies on unicellular organisms (Eckert, 1972; Machemer, 1974b) 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 (Girard & Kennedy, 1986). In mammalian respiratory 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 the ciliary activity of human respiratory cells.

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

9.3. Materials and methods

Sample collection. Ciliated nasal epithelium was obtained from the mucosa overlying the turbinates using a cytology brush (see page 49). Two or three brushings were taken from the same nostril of each subject. Cellular material adhering to the brush was dislodged in Eppendorf tubes containing 1 ml of tissue culture medium (Medium 199). All the experiments were performed within 4 hours 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 10 healthy volunteers. None of them had a history of upper respiratory tract infection for at least one month. The first group of subjects consisted of 6 men and 4 women, aged 23-50 years (mean 32.3 years); 2 of them were smokers. Samples obtained from these subjects were divided into two aliquots and used to test the effects of a control solution or 4-Br-A23187 (10⁻⁶ M) on CBF. The second group included 7 men and 3 women, aged 18-32 years (mean 25.8 years; 3 smokers). Cells from these subjects were used to measure the intracellular calcium concentration at baseline and after exposure to 10⁻⁶ M 4-Br-A23187. The third group of subjects consisted of 5 men and 5 women, aged 18-42 years (mean 27.6 years); 4 of them were smokers. Sample obtained from these subjects were used to study the effect of 10⁻⁴ M trifluoperazine
Measurement of CBF. Ciliated cells were transferred in Medium 199 to a perfusion chamber (see page 52). The chamber was connected to a perfusion pump delivering 0.25 ml/min, which gave a 90% wash-out of the fluid in the chamber in 10 minutes. CBF was measured at room temperature (23.1 ± 1.3°C) using the photometric technique (see page 55). In order to eliminate the increase in CBF induced by perfusion (Chapter 4.), all recordings were taken in the absence of flow. One cell from each preparation was studied throughout the experiment. The choice of the cell was randomized (see page 55). Following a 30 minute period of equilibration on the microscope stage, the sample in the chamber was perfused for 10 minutes with fresh Medium 199 equilibrated with room air. Baseline values were obtained by taking the mean of CBF measurements after 1, 15 and 30 minutes. The pH of all solutions tested was between 7.2 and 7.4. The extracellular calcium 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 perfusate.

Effect of calcium ionophore on CBF. Following the baseline readings, the perfusate was replaced with either Medium 199 containing the vehicle or Medium 199 supplemented with 4-Br-A23187 (10⁻⁶ M). The calcium ionophore was dissolved in dimethyl sulphoxide (DMSO) and both solutions contained final concentrations of 1% DMSO, a concentration
known not to affect CBF (Chapter 8.). CBF was measured after 1 minute (following the 10 minute period of perfusion) and then every 10 minutes for 1 hour.

**Effect of TFP on CBF.** 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 minute after perfusion and then every 15 minutes for 1 hour. After this period of observation, the perfusate was replaced with Medium 199 supplemented with 4-Br-A23187 ($10^{-6}$ M) and the CBF was measured after 1 minute (following the 10 minute period of perfusion) and then every 10 minutes for 1 hour.

**Measurement of intracellular free calcium concentrations.** Cells were loaded with the calcium indicator fura-2 by incubation with a membrane permeant form of the dye (1.0 $\mu$M acetoxymethyl ester) in Medium 199 for 30 minutes at 37°C in a shaking water bath. The cells were subsequently centrifuged, resuspended, washed twice and then plated onto Poly-l-lysine coated 32 mm square glass coverslips. The coverslips were mounted on the stage of a Nikon Diaphot inverted microscope and the cells were viewed at 400x using a Nikon 40x 1.3NA 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 narrowed to the area of the cell using an iris diaphragm to exclude stray or scattered light and to optimise the signal ratios. The cell was then illuminated alternately with 360 nm and
390 nm narrow band filtered (Ealing Electro-optics Filters, England) U.V. light from a 150W 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 milliseconds. The U.V. 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 digitisation by a CED 1401 Laboratory Interface (Cambridge Electronic Design, England). The same equipment also controlled the filter movement. The ratio of the emission signals at 360 nm and 390 nm was used to calculate the free intracellular calcium concentration, \([Ca^{2+}]_i\), from the equation:

\[
[Ca^{2+}]_i = K_d \left( \frac{F_q}{F_i} \right) \left( R - R_{\text{min}} \right) / \left( R_{\text{max}} - R \right)
\]

where \(R_{\text{max}}\) is the ratio value at saturating calcium, \(R_{\text{min}}\) the ratio value at limitingly low calcium, \(\left( F_q/F_i \right)\) 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 (Gryniewicz et al., 1985). These values were obtained by the method of Almers and 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 with either Medium 199 alone or Medium 199 supplemented with $10^{-6}$ M 4-Br-A23187. We used the more efficient 4-bromo form of the calcium ionophore (Debono et al., 1981) to avoid the fluorescence interference artifacts associated with the non halogenated form (Deber et al. 1981) and to optimise 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 seconds.

**Chemical reagents.** 1X Medium 199 (Flow Laboratories), DMSO, 4-Br-A23187, TFP dihydrochloride, and Poly-l-lysine (Sigma), fura-2 (Molecular Probes Inc.).

**Statistical analysis.** CBF values were expressed as the mean ± SEM and the response as the percentage changes from the baseline CBF. Differences between CBF values were determined by a paired Wilcoxon test; time-response relationships was determined by a Spearman rank correlation test. Significance was accepted when $P < 0.05$.

9.4. Results

**Effect of calcium ionophore on CBF.** The baseline CBF (7.1 ± 0.2 Hz) was unchanged after perfusion with control solution (Fig. 9.1.). In these
Fig. 9.1. Effect of control solution (triangles) and $10^{-5}$ M 4-bromo-calcium ionophore A-23187 (circles) on ciliary beat frequency (CBF) (mean ± SEM).

B = baseline;

$T_1$, $T_{10}$, $T_{20}$, $T_{30}$, $T_{40}$, $T_{50}$, $T_{60}$ = measurements 1, 10, 20, 30, 40, 50, 60 minutes after perfusion;

* $P < 0.01$ (versus baseline).
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 $6.8 \pm 0.2$ Hz (Fig. 9.1.). The rise in CBF was statistically significant 10 minutes after the end of the perfusion (+10%; $P < 0.01$) and continued to increase until 40 minutes (+18%; $P < 0.01$), with a plateau thereafter. At the end of the observation period (1 hour 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.* Perfusion of the cells with Medium 199 alone did not significantly affect the $[\text{Ca}^{2+}]_{i}$ (Fig. 9.2.). Exposure to $10^{-5}$ M 4-Br-A23187 caused a statistically significant increase of $[\text{Ca}^{2+}]_{i}$ (Fig. 9.2.) within 1 minute (+89 nM; $P < 0.01$). The $[\text{Ca}^{2+}]_{i}$ continued to rise up to 10 minutes (+450 nM; $P < 0.01$), after which there was a plateau which was sustained for at least 20 minutes.

*Effect TFP on CBF.* Perfusion with $10^{-4}$ M TFP caused a reduction in CBF with respect to a baseline of 7.2 ± 0.2 Hz (Fig. 9.3.). The decrease in CBF was statistically significant 30 minutes after perfusion (-7%; $P < 0.05$) and minimal CBF values were reached after 45 minutes (-10%; $P < 0.01$). At the end of the observation period (1 hour after perfusion) the CBF was still reduced relative to baseline (-9%; $P < 0.01$). Subsequent perfusion with $10^{-5}$ M 4-Br-A23187 did not change CBF (Fig. 9.3.)
Fig. 9.2. Effect of control solution (triangles) and $10^4$ M 4-bromo-calcium ionophore A23187 (circles) on the intracellular free calcium concentration ([Ca$^{2+}$]$_i$) (mean ± SEM).
Fig. 9.3. Effect of control solution (circles) and $10^{-4}$ M trifluoperazine (triangles) on ciliary beat frequency (CBF) (mean ± SEM).

B = baseline;
T_1, T_{15}, T_{30}, T_{45}, T_{60} = measurements 1, 15, 30, 45, 60 minutes after perfusion;
A23187 = measurement 40 minutes after perfusion with 4-bromo-calcium ionophore A23187 ($10^{-6}$ M);
* $P < 0.05$; ** $P < 0.01$ (versus baseline).
9.3.) and the CBF remained below baseline values (-10%; \( P < 0.01 \)) for the whole period of observation (1 hour). Incubation for 1 hour with control solution did not affect the expected rise following perfusion with \( 10^{-5} \text{ M} \) 4-Br-A23187 (+16% after 40 minutes; \( P < 0.01 \)) (Fig. 9.3.).

9.5. Discussion

*Calcium control of ciliary activity.* Calcium is an important regulator of cell function (Rasmussen, 1986ab) and is involved in the control of both flagellar (Tash & Means, 1983) and ciliary (Verdugo et al., 1983; Kakuta et al., 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, 1974b). In contrast, the lateral cell cilia of *Mytilus* gill are arrested by an increase in intracellular calcium (Satir, 1975). Several studies indicate that the 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 CBF.* In our study, addition of \( 10^{-5} \text{ M} \) 4-Br-A23187 led to an increase in CBF and this response has some similarities with that seen in rabbit tracheal explants (Girard & Kennedy, 1986; Sanderson & Dirksen, 1989). Girard and Kennedy (1986) challenged rabbit tracheal cells with \( 10^{-6} \text{ M} \) A23187 and observed an immediate increase in CBF with a gradual decrease after 20-30 minutes,
although CBF remained above initial levels for 1 hour. In our experiments, the rise in CBF had an onset between 1-10 minutes after challenge and reached its maximum after 40 minutes. The time-course of the calcium ionophore-dependent increase of CBF was different from that observed in our previous studies on the stimulation of CBF through cyclic AMP-dependent mechanisms (Chapter 8.), where 10⁻³ M dibutyryl cyclic AMP induced an immediate 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 and Kennedy (1986) in rabbit tracheal cells (+30%), it was similar to that observed in our previous study with dibutyryl cyclic AMP (+15%) (Chapter 8.).

**Effect of calcium ionophore on intracellular free calcium.** A23187 increases the uptake of ⁴⁵Ca²⁺ in several tissues (Reed & Lardy, 1972; Prince et al., 1973) and, at low concentrations, causes a release of Ca²⁺ from an intracellular Ca²⁺ pool (Babcock et al., 1976; Stolze & Schulz, 1980). In their study on rabbit tracheal cells, Girard and Kennedy (1986) investigated ⁴⁵Ca²⁺ uptake in the presence of A23187 and reported that kinetic analysis of the uptake curves showed two distinct exchangeable components, a fast component and a slow component. The authors suggested that the fast component might reflect movement of Ca²⁺ across the plasma membrane, while the slow component might be associated with intracellular Ca²⁺ sequestering organelles such as the mitochondria or endoplasmic reticulum.
In our study, we calculated \([\text{Ca}^{2+}]\) using values obtained by the method of Almers and 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 giga ohm 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 structures 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, absolute values quoted can only be taken as best available estimates. However, the relative changes in calcium concentration would be unaffected by the lack of absolute calibration standards.

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

**Effect of TFP on CBF.** 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 and 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 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 (1983) on rabbit cells. They observed an increase in the effect of Ca\(^{2+}\) on the CBF of demembranated tracheal cilia by adding exogenous calmodulin. However, they also reported that TFP-induced inhibition of CBF could be reversed by exposure to A23187, a finding not confirmed in our study. Indeed, the
complete abolition of the cilio-stimulatory effect of 4-Br-A23187 in the presence of TFP 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\textsuperscript{2+}-dependent protein kinase (PKC) (Schatzman et al., 1981) in addition to calmodulin-dependent enzymes. Therefore, it remains 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 man (Chapter 8.). In these studies, we showed that a range of concentrations of H-7 (10\textsuperscript{-6} to 10\textsuperscript{-3} M), an inhibitor of cyclic nucleotide-dependent kinases and of PKC (Hidaka et al., 1984) did not affect the basal CBF, an observation reported also by others in rabbit tracheal explants (Kobayashi et al., 1988). H-7 (10\textsuperscript{-4} and 10\textsuperscript{-3} M), however, was able to block a cyclic AMP-dependent increase in CBF. We also demonstrated that stimulation of PKC with 10\textsuperscript{-5} M phorbol myristic acid (PMA) did not change CBF. The combination of the results on the effects of H-7, PMA 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 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 PKC-dependent enzymes had no effect on CBF (Chapter 8.), 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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Full papers

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