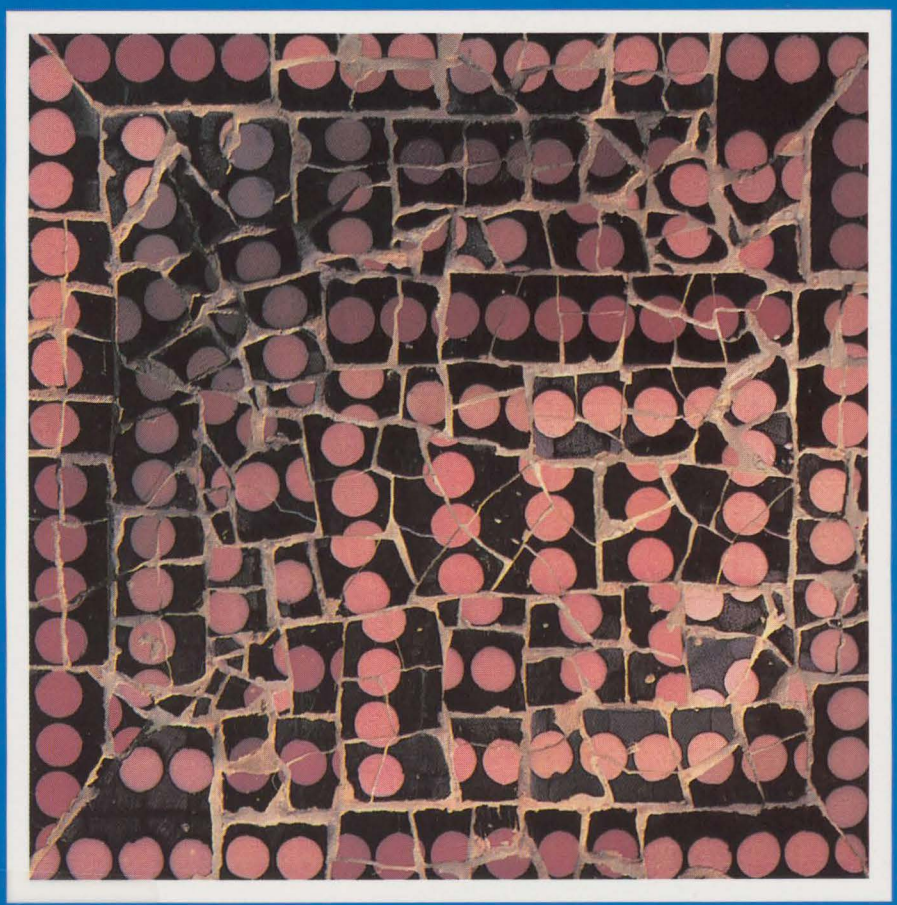
EFFECTS OF MEDIATORS AND NEUROPEPTIDES ON HUMAN UPPER RESPIRATORY CILIA



P.J. SCHUIL

STELLINGEN

behorend bij het proefschrift

EFFECTS OF MEDIATORS AND NEUROPEPTIDES ON HUMAN UPPER RESPIRATORY CILIA

Universiteit Utrecht
dinsdag 13 december 1994

PAULUS JOHANNUS SCHUIL

1. Een aantal belangrijke mediatoren van type I allergie, zoals histamine, leukotriene C_4, prostaglandine D_2 en prostaglandine E_2, heeft in vitro geen direct negatief effect op de activiteit van trilharen van de menselijke bovenste luchtwegen.

2. Prostaglandine E_2 en calcitonin gene-related peptide hebben in vitro een stimulerende invloed op de trilhaaractiviteit in epitheel van de menselijke bovenste luchtwegen.

3. Mediatoren van type I allergie en neuropeptiden, die de trilhaaractiviteit in vitro stimuleren, bewerkstelligen mogelijkerwijs een verhoogd mucociliair transport in vivo. Hierdoor kan het afweermechanisme van de luchtwegen versterkt worden.

4. In onderzoek naar het effect op de functie van trilharen, waarbij stoffen worden getest die klinisch van belang zijn, verdient het gebruik van humaan trilhaarepitheel de voorkeur.

5. Bij hyperreactiviteit van de luchtwegen speelt een aantal in het slijmvlies vrijgemaakte neuropeptiden een rol.

6. Er zijn aanwijzingen dat passief roken de kans verhoogt op het optreden van onder meer luchtwegaandoeningen en otitis media met effusie. Daarom is het verontrustend dat in een recent Nederlands onderzoek* werd gevonden dat van een groep jonge kinderen bijna de helft binnenshuis aan tabaksrook bleek te worden blootgesteld.
   *Hirasing RA et al., Ned Tijdschr Geneeskd 1994; 28: 1422-1426.

7. Ook in gevallen waarbij na een acuut ontstaan enkelzijdig perceptief gehoorverlies ("sudden deafness") weer verbetering optreedt, is het niet uitgesloten dat zich aan de aangedane zijde een brughoekproces bevindt.

8. De sublabiale rhinotomie ("midface degloving") is een chirurgische benadering die in de meeste gevallen meer mogelijkheden biedt dan de laterale rhinotomie en bovendien betere cosmetische resultaten geeft.

9. Het appliceren van rode peper in de neus zou de symptomen van nasale hyperreactiviteit kunnen doen verminderen.

10. Bij een promotie is het essentieel data te reduceren tot een datum.

EFFECTS OF MEDIATORS AND NEUROPEPTIDES ON HUMAN UPPER RESPIRATORY CILIA

Cover illustration: Mosaic by Antoni Gaudí, Park Güell, Barcelona.

EFFECTS OF MEDIATORS AND NEUROPEPTIDES ON HUMAN UPPER RESPIRATORY CILIA

EFFECTEN VAN MEDIATOREN EN NEUROPEPTIDEN
OP TRILHAREN VAN DE
MENSELIJKE BOVENSTE LUCHTWEGEN

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr. J.A. van Ginkel,
ingevolge het besluit van het College van Dekanen
in het openbaar te verdedigen
op dinsdag 13 december 1994 des namiddags te 2.30 uur

door

PAULUS JOHANNUS SCHUIL

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Hisce igitur feliciter peractis

Voor Marja
Paul en Bart
Aan mijn ouders

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Cover illustration from "Antoni Gaudí" by Rainer Zerbst. Courtesy of Benedikt Taschen Verlag.

CONTENTS

LIST OF ABBREVIATIONS

ANOVA	:	analysis of variance
CGRP	:	calcitonin gene-related peptide
CBF	:	ciliary beat frequency
FFT	:	fast Fourier transform analysis
LTC_4	:	leukotriene C_4
PGD_2	:	prostaglandin D_2
PGE_2	:	prostaglandin E_2
SC	:	signal consistency
SP	:	substance P

CHAPTER 1

INTRODUCTION AND OBJECTIVES

INTRODUCTION

Billions of cilia lining the airways provide the driving force of the mucociliary transport in the respiratory tract. Mucociliary transport is a key element in the defense of the airways. Besides ciliary activity, mucus is also a major factor in mucociliary clearance. The importance of this defense mechanism becomes evident when either ciliary activity or mucus production is impaired. This occurs, for example, in primary ciliary dyskinesia and cystic fibrosis, respectively. Such defects may lead to chronic airway infections.

Ciliary activity can be characterized by the ciliary beat frequency (CBF). Alterations in CBF might lead to changes in mucociliary transport. This, in turn, may interfere with the defensive role of mucociliary clearance. The importance of the mucociliary transport system has not been completely determined yet. Besides, it is largely unknown how ciliary activity can be influenced.

The mucociliary transport can be impaired in allergic subjects. In allergic rhinitis and asthma the symptoms may originate from this disturbed mucociliary transport. The mediators released in a type I allergic reaction play an important role in the pathogenesis of allergy. It is not clear to what extent mediators of allergy influence the ciliary activity. Therefore, further study is needed to elucidate precisely how these mediators affect the functioning of cilia in the human respiratory tract.

In patients with hyperresponsiveness of the airways, several neuropeptides are liberated in the respiratory mucosa. Little is known about the effects these substances have on ciliary activity.

OBJECTIVES

The main objective of this thesis is to determine the effects of a number of mediators of allergy and neuropeptides on the activity of cilia taken from the human upper respiratory tract. The in vitro technique developed in our department by Ingels was used to study these effects [1].

This thesis is based on the following investigations.

Chapter 2 presents a survey of the literature on ciliary activity of the upper respiratory tract in various circumstances. We also review the body of research into the effects that mediators of allergy and neuropeptides have on mucociliary function.

Chapter 3 explores the suitability of diverse sources of human ciliated epithelium for ciliary function experiments. In that study, cell suspension cultures containing preserved epithelium, derived from nasal polyps and inferior turbinates, and fresh epithelium, obtained from adenoids, are investigated.

Chapter 4 describes how 2 mediators of allergy, histamine and leukotriene C_4, influence ciliary activity.

Chapter 5 deals with the effects of the mediators prostaglandin D_2 and prostaglandin E_2 on ciliary activity.

These mediators of allergy were chosen because they can be demonstrated in elevated concentrations in nasal secretions of allergic subjects after allergen challenge. Moreover, these mediators are considered to be relevant in the pathogenesis of allergic rhinitis, as they are able to induce part of the symptoms of allergic rhinitis.

Chapter 6 reports on the way the neuropeptide substance P affects ciliary activity.

Chapter 7 describes the effects of the neuropeptide calcitonin gene-related peptide on ciliary activity.

We investigated these 2 neuropeptides because they are released locally from sensory nerve endings in the human nasal mucosa, and are thought to play a role in hyperresponsiveness of the airways to nonspecific stimuli.

Finally, in *Chapter 8*, the results of this study are summarized and discussed.

REFERENCE

1. Ingels, KJAO. Ciliary beat frequency and harmony in the human nasal mucosa. Thesis 1991, Utrecht University, the Netherlands.

CHAPTER 2

MORPHOLOGY, PHYSIOLOGY AND PATHOLOGY OF THE UPPER RESPIRATORY MUCOSA, WITH SPECIAL EMPHASIS ON CILIA

A review of the literature

MORPHOLOGY OF THE UPPER RESPIRATORY MUCOSA

The epithelium that lines the airways is mostly of a pseudostratified ciliated columnar type (Figure 1). This epithelium is the airways' first line of defense against inhaled infectious or toxic agents. It covers about 0.5 m^2 in human beings [31]. In the upper airways, this kind of epithelium is found in several locations: the nasal cavities; the paranasal sinuses; the nasopharynx including the adenoid surface; parts of the larynx; and the Eustachian tube and middle ear [42,100,114,179].

This epithelium contains various cell types: ciliated and non-ciliated columnar cells; mucus-producing goblet cells; and basal cells [100,114] (Figure 2). The basal cells do not reach the surface of the epithelium. All cells have contact with a basement membrane, which divides the epithelium from the subepithelial layer or lamina propria ('submucosa'). The height of the epithelium is about 25 µm. At their apical surface, the non-ciliated columnar and goblet cells have 300-400 microvilli. As the name implies, the ciliated columnar cells also have cilia. The number of cilia may vary throughout the respiratory tract from 50 to 250 on a single ciliated cell. The lamina propria consists of connective tissue, which contains nerves, blood vessels, and numerous seromucous glands. These glands have tubules with orifices distributed over the epithelium.

A layer of mucus lies on top of the epithelium. It is composed of 2 sheets with different composition. The cilia are embedded in a serous fluid, the so-called periciliary or sol layer, which is covered by a gel layer with higher viscosity. Already in 1934, Lucas and Douglas assumed the presence of these 2 different mucus layers [85]; electron-microscopic studies eventually confirmed their presence [9,139]. The mucus blanket varies in thickness between 0.5 and 10 µm. It is produced mainly by the seromucous glands, though the goblet cells also contribute [72]. These secretions are thought to be extruded through the periciliary fluid to the surface [116]. Here they are subjected to the physical properties of the respiratory gases. Furthermore, secretions can be derived from the vascular bed because of increased permeability. The microvilli at the epithelial surface are thought to play a role in maintaining homeostasis of the periciliary fluid layer [114].

CILIA AND THEIR STRUCTURE

Phylogenetically, cilia are very old structures and can be found in almost all species, from protozoa to mammals. Wherever they are found, cilia have about the same structure and function [31]. The Dutchman Antonius de Heide

is generally credited with the first description of cilia in mussels in 1684 [59]. However, his countryman Antony van Leeuwenhoek had already mentioned these structures in 1676 [59].

In man, cilia occur not only in the airways. For instance, in the central nervous system, they are seen in the ependymal lining of the brain and the central spinal canal. In the genital tract, they are present in the efferent ductules of the epididymis, the fallopian tube and fimbriae, as well as in deeper parts of the uterine cervix. Furthermore, a cross-section of the human sperm tail reveals an ultrastructure that resembles the one found in a cilium [3].

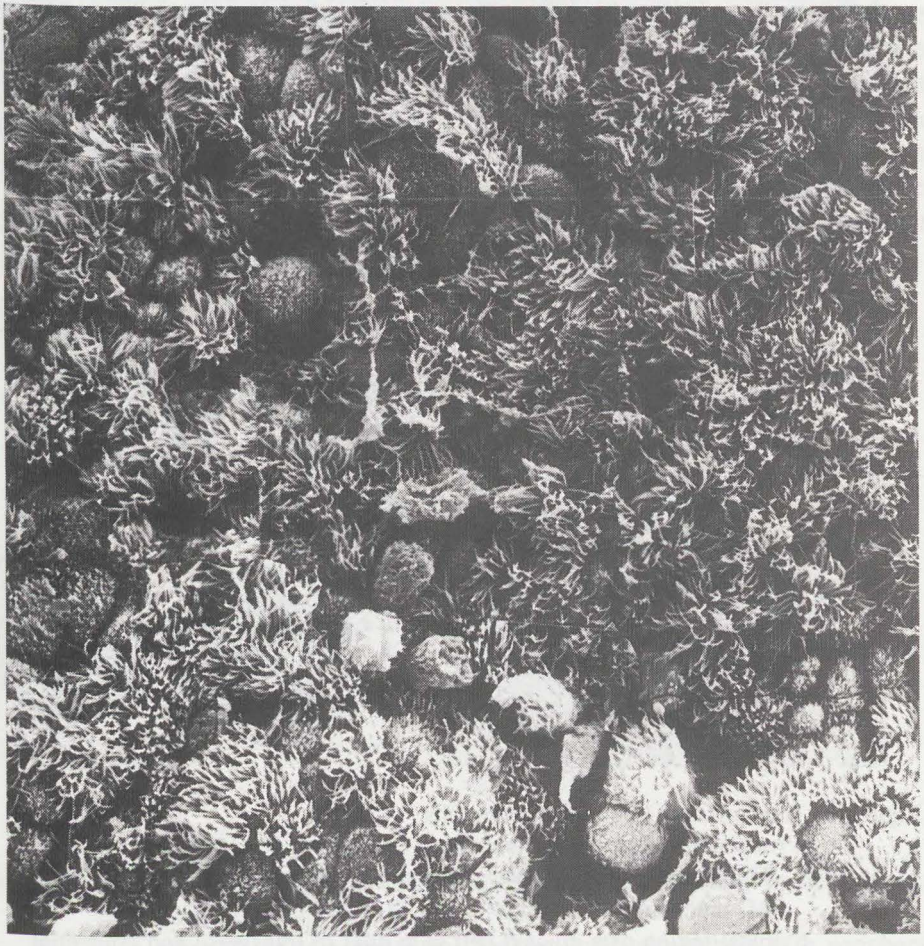

Figure 1 Scanning electron micrograph illustrating the surface of the pseudostratified ciliated columnar epithelium.

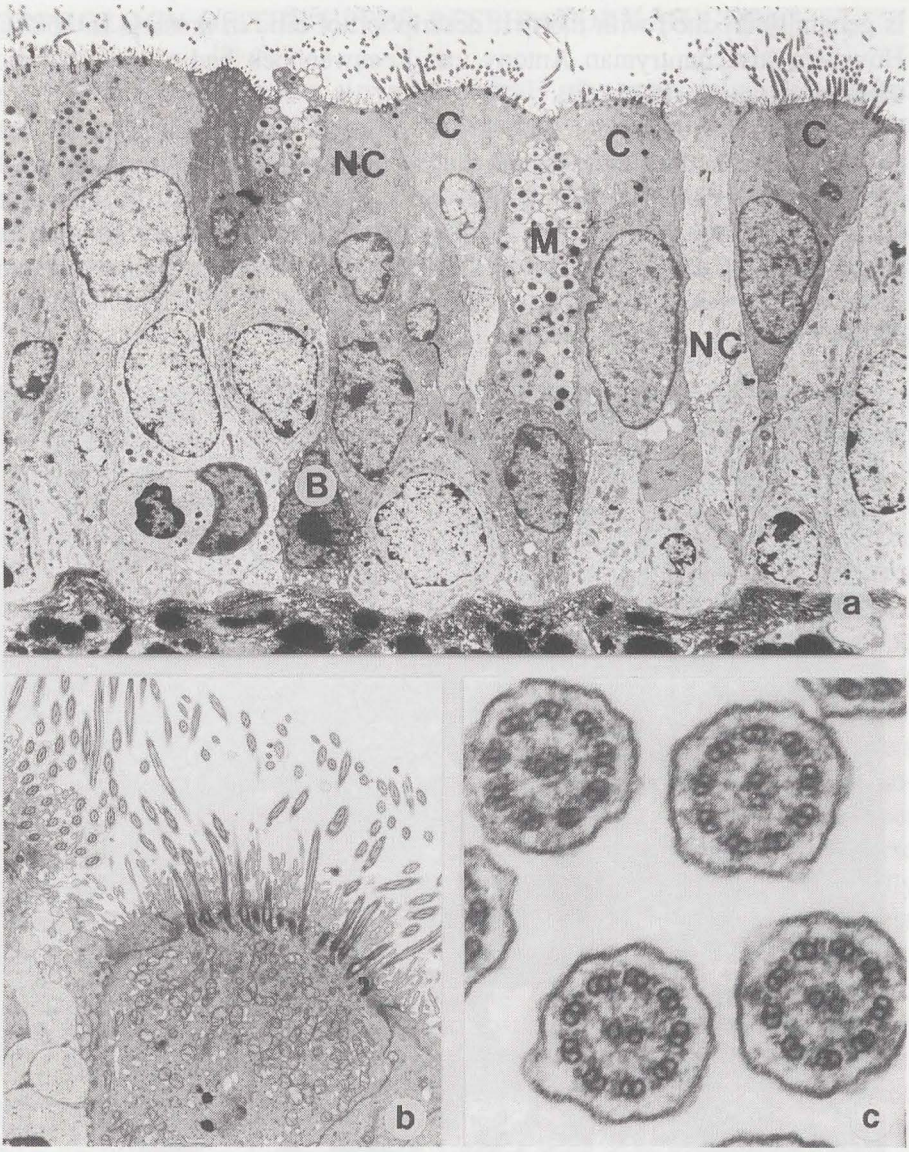

Figure 2 Transmission electron micrographs.

a pseudostratified ciliated columnar epithelium (x2,100)
C: ciliated columnar cells; NC: non-ciliated columnar cells
M: mucus-producing goblet cell; B: basal cell

b apical region of a ciliated cell with cilia in transverse and tangential section (x4,800)

c cross-section of cilia demonstrating their ultrastructural morphology (x95,400; see also Figure 3).

Current data on the ultrastructure of cilia has largely been gathered by electron-microscopic research [35,37,69,142]. The length of a cilium in the respiratory tract is about 5-7 µm, but this size differs according to the species. The shaft of a cilium is the protrusion at the epithelial surface. It consists of an axonema and a surrounding cell membrane. The shaft has a diameter of 0.2-0.3 µm. Furthermore, a cilium has a basal body and basal roots. Figure 2c shows several cilia in cross-section; the ultrastructural morphology of the axonema is schematically depicted in Figure 3. The axonema demonstrates a characteristic '9+2' pattern of microtubules; there are 9 pairs of peripheral doublets of microtubules and 2 central single ones. A peripheral pair is composed of an A-microtubule, with 13 filaments, and a B-microtubule, with 11 filaments [158]. The microtubules contain the globular protein tubuline. An inner and outer dynein arm originate from the peripheral A-microtubule. They make periodic contact with the B-microtubule in a ciliary movement. The dynein arms contain 2 enzymes with ATPase activity. Spokes are found between the A-microtubules and the central sheath surrounding the central microtubules. ATPase activity has also been shown in the heads of the spokes [105]. A peripheral doublet is connected with both adjacent ones by nexin links.

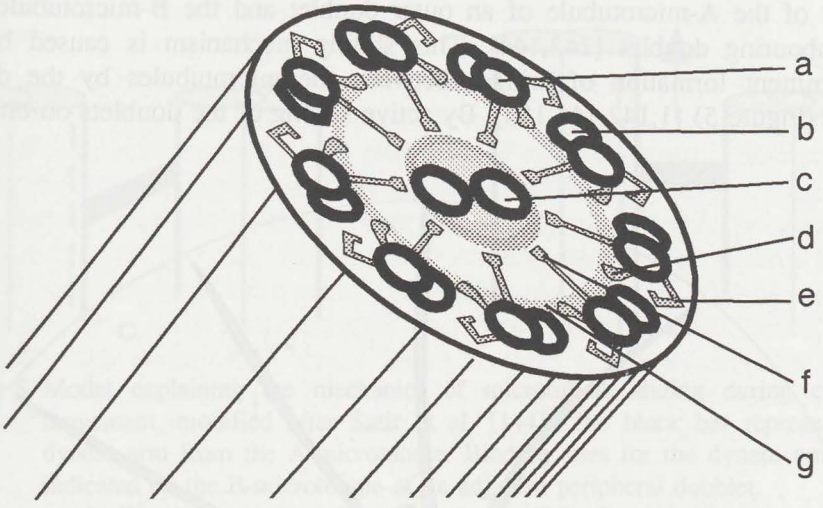

Figure 3 Schematic representation of the ultrastructural morphology of an axonema in cross-section.
a: A-microtubule of a peripheral doublet; b: B-microtubule of a peripheral doublet; c: central microtubule; d: inner dynein arm; e: outer dynein arm; f: radial spoke; g: nexin link.

CILIARY PHYSIOLOGY

The ciliary activity is the driving force in the mucociliary transport. Cilia in the respiratory tract have a beat frequency of about 8-20 Hz, varying between species and individuals [147]. The ciliary beat frequency (CBF) constitutes an important parameter of this ciliary activity [33,122]. In the same individual, nasal, tracheal, and bronchial CBF do not differ [131]. In subsegmental airways, on the other hand, CBF is significantly lower [131].

The ciliary movement consists of an effective stroke, a recovery stroke, and a rest phase. This movement was described in detail by Satir in 1974 [143]. In the effective stroke, the cilium is totally stretched, and its tip just reaches the gel layer. Next, the cilium is curved, bending back in a plane parallel to the epithelial surface towards its starting position, where it enters a rest phase (Figure 4). Adjacent cilia move during their effective stroke in the same direction, in a more or less co-ordinated manner. This leads to a sequence of cilia moving behind each other. That sequential movement, called metachronal co-ordination, seems to be based on mechanical impulses from one cell to another. These co-ordinated ciliary beats transport mucus, especially the gel layer, in one direction.

The movement of a cilium is thought to originate from sliding along each other of the A-microtubule of an outer doublet and the B-microtubule of a neighbouring doublet [143,144]. This sliding mechanism is caused by the intermittent formation of bridges between the microtubules by the dynein arms (Figure 5) [1,142,144,153]. By active sliding of the doublets on one side

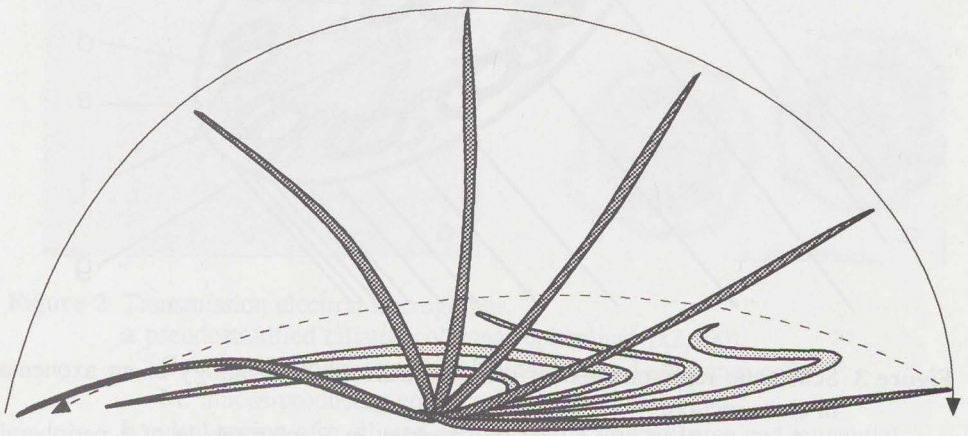

Figure 4 Drawing demonstrating the different planes of the effective and recovery stroke during ciliary movement.

of the axonema, the cilium is bent in one direction, whereas active sliding by doublets located on the other side lets the cilium bend back again [147]. In addition, the radial spokes contribute to the bending movement, as the spoke heads are able to make selective intermittent connections with the central sheath [174]. In this way, they also help resist the sliding movement. At present, it is unknown how the selective attachment and reattachment of the dynein arms and the radial spokes is coordinated within the axonema. The energy for the ciliary movement is delivered by ATP. As the dynein arms contain ATPase, they are able to hydrolyse the ATP. Mg^{2+} is essential for this hydrolysis. Moreover, an increase in intracellular Ca^{2+} or a rise in cyclic AMP stimulates the cilia to beat at a higher frequency [156,169].

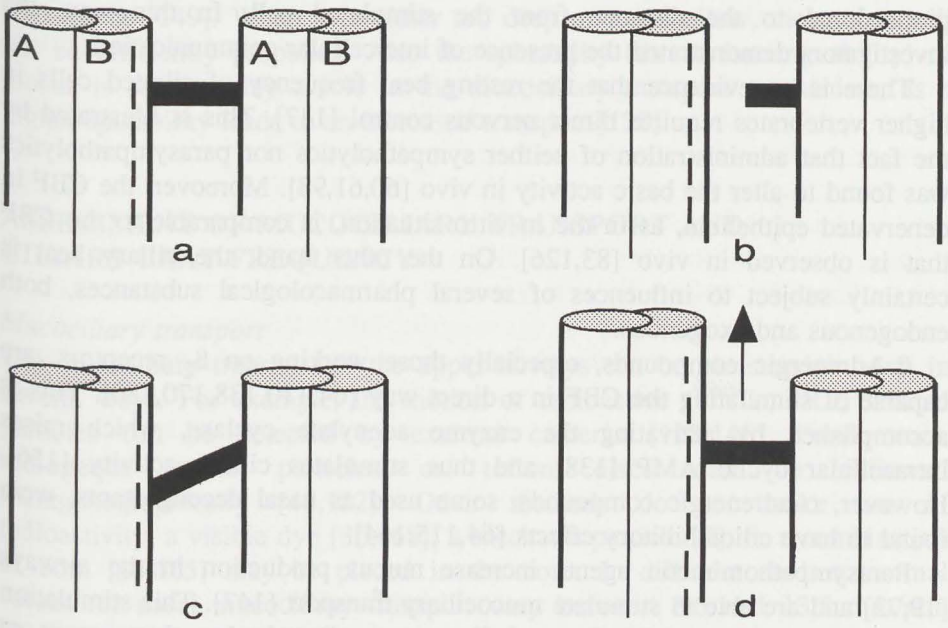

Figure 5 Model explaining the mechanics of microtubule sliding during ciliary movement, modified after Satir et al. [144]. The black bar represents a dynein arm from the A-microtubule. Binding sites for the dynein arm are indicated on the B-microtubule of an adjacent peripheral doublet.

- **a** the dynein arm is bound to a distal site on the B-microtubule
- **b** release and shortening of the dynein arm
- **c** re-extension and reattachment to a more proximal site on the B-microtubule
- **d** return to its original position causing sliding of the A- and B-microtubules.

INFLUENCES ON CILIARY BEAT

The CBF is dependent upon temperature. A rise in temperature between 20 and 40 °C increases CBF in a linear way [63,92]. When kept in a certain range, pH (6.5-9.0) and osmolarity (300-450 mosm/l) do not affect in vitro CBF in different species [63,87,166], whereas depression of CBF does occur beyond these values. Those limits are probably set by the periciliary sol layer, as a way to maintain an optimal CBF.

Sanderson et al. demonstrated that after mechanical stimulation of ciliated cells with a fine needle, their beat frequency was higher [136,138]. This was thought to be caused by a rise in intracellular Ca^{2+}. Adjacent ciliated cells also reacted with an increase in beat frequency, though with a delay proportional to the distance from the stimulated cell. In this way, the investigators demonstrated the presence of intercellular communication.

There is no evidence that the resting beat frequency of ciliated cells in higher vertebrates requires direct nervous control [147]. This is illustrated by the fact that administration of neither sympatholytics nor parasympatholytics was found to alter the basic activity in vivo [60,61,93]. Moreover, the CBF in denervated epithelium, as in the in vitro situation, is comparable to the CBF that is observed in vivo [83,126]. On the other hand, the ciliary beat is certainly subject to influences of several pharmacological substances, both endogenous and exogenous.

ß-Adrenergic compounds, especially those working on $ß_2$ receptors, are capable of stimulating the CBF in a direct way [64,110,138,170,180]. This is accomplished by activating the enzyme adenylate cyclase, which raises intracellular cyclic AMP [138] and thus stimulates ciliary activity [156]. However, α-adrenergic compounds, some used as nasal decongestants, were found to have cilioinhibitory effects [64,115,164].

Parasympathomimetic agents increase mucus production in the airways [19,72] and are able to stimulate mucociliary transport [147]. This stimulation is probably brought about in an indirect way. By altering the amount or composition of mucus, they may indirectly affect ciliary activity. Moreover, there is no convincing evidence that the ciliary beat in the airways is under direct cholinergic influence [64,147,180].

MUCUS PROPERTIES

In the upper airways, mucus is led from the nasal cavities and sinuses to the pharynx, where it is swallowed [53,94,95,185]. In the lower airways, mucus is transported from the peripherial bronchioles upwards to the pharynx [3]. In

this way, mucus-entrapped toxic or infectious particles are removed from the airways.

It is still a matter of debate in the literature to what extent mucus properties may influence mucociliary transport and ciliary beat [145]. Several in vitro studies show that increased viscosity and elasticity of mucus decrease mucociliary transport and CBF [46,71,87,119]. At low viscosity values, an increase in viscosity leads to a sharp decline in CBF [71,87]. At higher viscosity levels, an increase brings about a slight drop in CBF [71]. It is conceivable that viscosity fluctuations in the periciliary sol layer may inhibit mucociliary transport or beat frequency more strongly than changes in the gel layer would.

Furthermore, the depth of the periciliary fluid layer is thought to be of importance [149]. When it is either too deep or too shallow, the mucus will not be efficiently propelled. Also the spinability (thread-forming ability) of mucus is said to play a role in the mucociliary transport mechanism, as a higher spinability leads to an increased transport [120].

ASSESSMENT OF MUCOCILIARY TRANSPORT AND CILIARY BEAT FREQUENCY

Mucociliary transport

The mucociliary transport of the upper airways can be measured in vivo in several ways. For example, the motion of radioactive (^{99m}Tc, ^{51}Cr) labeled particles can be detected by external cameras [123,146]. Furthermore, radiopaque metal particles or teflon discs can be followed roentgenographically [43,132]. Other methods do not make use of radioactivity: a visible dye [36,168], a saccharin particle [8], or a combination of both [33,165] may be placed in the nose. In different studies, normal values for nasal mucociliary transport vary between 3.6 and 13.5 mm/min [73,146]. The mucociliary clearance from the lower airways can be determined by using a gamma camera to monitor how an inhaled aerosol, with radioactive-labeled particles, disappears from the tracheobronchial tree [22]. A more or less comparable way was described by Cohen et al. [28], who applied magnetic dust (Fe_3O_4) in combination with a magnetometer.

Ciliary beat frequency

The CBF can be measured in 2 different ways: by the reflected light or the transillumination technique. In 1933, Lucas reported that microscopic light was reflected as a result of the ciliary activity [86]. This principle is still used. These days, the technique can be carried out by means of a laser beam

[169,181,182]. It can be applied to in vivo as well as in vitro experiments. In vivo studies using this technique were carried out by Hybbinette, Mercke, and Lindberg [62,84]. In these experiments, the ciliary beat was measured through a window in the anterior aspect of the maxillary sinuses of rabbits. After intra-arterial injection of agents, the changes in ciliary activity were determined. Reimer et al. reported in vivo measurements in patients undergoing a Caldwell-Luc procedure [125,126]. These investigators encountered many technical difficulties, however. Recently, in vivo measurement of CBF in the human trachea and bronchi with a fiber-optic bronchoscope was described [58]. The same technique was also applied to the human nasal mucosa by Lindberg and Runer [83]. The main advantage of the reflected light technique is that measurements can be carried out in vivo under physiological conditions. However, pharmacological studies are not yet well feasible in human subjects with this technique. Besides, changes in quality and quantity of mucus may alter the CBF in an indirect way. Thus, these experiments cannot distinguish between an indirect or direct effect on CBF. For this reason, authors using this technique state that 'mucociliary wave frequency' or 'mucociliary activity' is measured.

Another method to determine ciliary activity involves transillumination by microscopic light of a row of cilia from a specimen of respiratory epithelium [27,29,129,184]. This is the technique that is most often used. However, it can only be applied in vitro. Its advantage is that in pharmacological studies, it allows the effects of changes in mucus amount or composition on CBF to be circumvented.

The first methods to record the light signals that were altered by the cilia in reflecting or in transillumination techniques were conducted by Gray in 1930 and Proetz in 1933 [49,117]. These early studies made use of high-speed cinematography. The CBF can be measured on a screen when the film is projected. At present, video equipment is used for this purpose [124,127]. Dalhamn and Rylander's 1962 study was the first to use a photoelectrical cell to detect the variations in light intensity caused by the movements of the cilia [29]. CBF can be calculated from the electrical signals thus obtained with the reflected light as well as with the transillumination technique. The light variations caused by the moving cilia reveal a complex sinusoidal signal. The signal is the result of the action of several hundred cilia, the actual number depending on the area being measured. A fast Fourier transform (FFT) analysis of the electric signal was introduced for calculation of the CBF [65,118,137].

The metachronal co-ordination of cilia is essential for an efficient mucociliary transport. However, little attention has been given to the problem of determining co-ordination. Nevertheless, Wanner et al. estimated ciliary

co-ordination in a subjective manner, while measuring CBF [173]. Ingels et al. postulated a parameter for the ciliary beat harmony: the so-called signal consistency [66]. They calculated this parameter from the measured variations in light intensity in an objective way. This parameter was found to be independent of CBF. Therefore, it was supposed to be exclusively related to ciliary co-ordination. Gilain et al. analyzed ciliary beating heterogeneity from the power spectrum obtained by FFT [47]. The metachronal wave period was measured by Wong et al. with double-beamed laser equipment [182].

MUCOCILIARY PATHOLOGY

Ciliary dyskinesia

Various ultrastructural and functional abnormalities in cilia and spermatozoa tails are present in patients with primary ciliary dyskinesia, which is an inherited disorder [148,163,167]. The ultrastructural abnormalities may include absent or defective inner and/or outer dynein arms, absent radial spokes, microtubular transposition, or disorientation of the central pair of microtubules [21,167]. In addition, abnormal ciliary motility with a normal ultrastructure may be found [101]. The first descriptions of the ultrastructural defects were published in 1975 by Afzelius et al. [5], and by Pedersen and Rebbe [112]. Independently, these investigators observed the absence of dynein arms in the tails of immobile spermatozoa of 3 patients. One of these patients also showed all signs of the Kartagener syndrome - chronic sinusitis, bronchiectasis, and situs inversus. The second patient suffered from chronic sinusitis and bronchitis, while the third only had situs inversus. Subsequently, in 1976 the absence of dynein arms in respiratory cilia was demonstrated in patients with the Kartagener syndrome [2,111]. Thus, a common ultrastructural defect of cilia and spermatozoa tails was found. At first, the disorder was called immotile cilia syndrome [34]. However, as affected individuals can have motile cilia with an abnormal beating pattern, the term primary ciliary dyskinesia was proposed [148,163].

Due to the ciliary motility disorder, mucociliary transport does not occur. Chronic infections of the upper and lower respiratory tract may result. Furthermore, male patients are subfertile because of the spermatozoal defects. Recently, a possible genetic basis for certain forms of the disorder was demonstrated [171]. ATP and ATPase were able to enhance the ciliary motility in nasal biopsies of patients with this syndrome [41].

An overlap between primary ciliary dyskinesia and Young's syndrome was suggested [150]. Patients with Young's syndrome have sinusitis, bronchitis

with or without bronchiectasis, and an obstructive azoospermia. Their respiratory and ependidymal cilia are normal, as are their sperm tails. Hence, malfunction of the microtubules might be the basic abnormality [51].

In addition to primary ciliary disorders, there are acquired ones as well. Secondary ciliary dyskinesia is a disorder whereby ultrastructural abnormalities of the cilia develop as a result of respiratory infections. These deformations may take various forms: an abnormal number of central or peripheral microtubules, differing from the '9+2' pattern; or bleb-like and compound cilia [4,21,52,167].

Cystic fibrosis

Besides ciliary defects, changes in mucus can affect the mucociliary system too. For example, this occurs in patients with cystic fibrosis. In this disease, an abnormally viscous mucus is produced as a result of a defective regulation of Cl^--transport and an accelerated sodium reabsorption [30]. The nasal, tracheal, and pulmonary mucociliary clearance is decreased [76,130,183], which contributes to the development of chronic airway disorders. The ultrastructure and ciliary activity are normal, however [128,130].

Infectious disorders

While primary mucociliary defects can lead to chronic airway infections, these infections in turn can be responsible for impairment of the mucociliary transport system. Several investigations have demonstrated that mucociliary clearance in the nose [130,135] and the lower airways [48,121] may be decreased in patients with chronic sinusitis and bronchitis, respectively.

In about 25% of the chronic sinusitis cases, in vitro examination of nasal biopsies revealed no ciliary motility [21,104]. This may be due to the fact that normal ciliated epithelium has undergone squamous metaplasia [40,106]. Another factor could be a decrease in CBF. Some studies support this hypothesis [106,107]; in others, it could not be confirmed [104]. Certain bacteria - for example Haemophilus influenzae, Pseudomonas aeruginosa, Staphylococcus epidermidis - and bacterial endotoxins are capable of depressing CBF [38,176,177]. This was also found for Klebsiella ozaenae, which might contribute to the pathogenesis of atrophic rhinitis [39]. The changes in mucus properties that occur in the infectious state will also contribute to a decreased mucociliary transport [88,121]. Furthermore, neutrophil granulocytes produce proteolytic enzymes that are able to reduce ciliary beat [154,157].

It has been known for quite a long time that viruses causing rhinitis (common cold) may destroy ciliated cells [54,57]. In healthy volunteers inoculated with rhinovirus, nasal mucociliary transport was found to be

depressed [133]. This was also demonstrated in the chicken, where it appeared to be accompanied by a reduction in ciliated cells and CBF [134]. When mucociliary transport is diminished artificially in this model, the duration and severity of the viral infection is increased [161]. In patients with a naturally acquired common cold, decreased transport was measured for more than one month, together with a lower number of ciliated cells and a decline in CBF [113]. Furthermore, virus-induced ultrastructural defects in cilia may occur [23,178]. On the other hand, it should be kept in mind that the incidence of the common cold is not higher in patients with primary ciliary dyskinesia than among normal individuals [101].

Allergic rhinitis

Patients with an allergic rhinitis demonstrate a decreased mucociliary transport [50,91]. CBF was reported to be lower in these patients than in normal individuals [109]. Furthermore, CBF was reduced in an allergic-rhinitis group after nasal allergen challenge, whereas no change occurred in non-allergic controls [56]. Patients with nasal allergy who showed a good clinical response to immunotherapy for one year demonstrated higher CBF than before treatment [108]. In the nasal allergic reaction (type I, IgE-mediated), several mediators of allergy are released from the mucosa [20,102,103]. It is conceivable that they directly depress the ciliary beat and in this way diminish mucociliary clearance. Moreover, patients with asymptomatic allergic asthma have a lower tracheal mucociliary clearance when compared with normals, and they demonstrate a further decrease after bronchial allergen provocation [96]. Their clearance decline can be prevented by pretreatment with cromolyn sodium; this is indicative of the role that mediators of allergy play in depressing the mucociliary transport. Also changes in mucus production and composition may alter mucociliary clearance. In nasal secretions of subjects allergic to grass pollen, a significantly higher viscosity is found after nasal allergen challenge than after provocation with metacholine [19]. Furthermore, histological changes in epithelial cells as a result of the allergic reaction may add to a depressed mucociliary function. Indeed, electron-microscopic examination of nasal epithelium after allergen challenge reveals damage to the ciliated cells and shows their replacement by goblet cells [26,175].

These data suggest that functional disorders of the ciliated nasal epithelium, like impairment of mucociliary clearance and CBF, can occur in allergic rhinitis.

Non-allergic rhinitis

The term non-allergic rhinitis is used for a group of patients characterized by chronic nasal complaints and nonspecific nasal hyperresponsiveness that are not IgE-mediated allergic reactions [67,159]. Little is known about possible mucociliary function disorders in this condition.

A subgroup of these patients has eosinophilia in nasal smears (non-allergic rhinitis with eosinophilia syndrome, NARES) [68], which could be a preceding stage of the aspirin idiosyncrasy [98]. The latter is manifest as hypersensitivity to acetylsalicylic acid and non-steroidal antiinflammatory drugs. This response is frequently accompanied by attacks of asthma, nasal polyposis, and chronic sinusitis, possibly resulting in mucociliary disorders.

A second subgroup of patients presents the symptoms of classic rhinitis medicamentosa: nose complaints because of their longstanding abuse of nasal decongestants (α-adrenergics). These substances are known for their cilioinhibitory effect. Abuse could lead to decreased mucociliary transport.

In a third subgroup, nasal symptoms are associated with physical and chemical exposure. Rabbit maxillary sinuses show an increased in vivo mucociliary activity when subjected to cigarette smoke and ammonia, possibly indicating a reflex mechanism [80]. On the other hand, an earlier study revealed depression of ciliary activity [74]. However, nasal mucociliary clearance time in smokers is twice as long as in non-smokers, although their CBF did not differ in nasal biopsies [151].

Several immunohistochemical studies have demonstrated the presence of neuropeptides in the human nasal mucosa [7,10-13,160]. Neuropeptides like substance P (SP), calcitonin gene-related peptide (CGRP), and neurokinin A (NKA) are present in sensory trigeminal nerve endings. They are released locally into the nasal epithelium on trigeminal nerve stimulation, for example by nonspecific physical, thermal or chemical stimuli. Furthermore, a reflex pathway is initiated. This leads to centrally activated reflexes like itching, sneezing, and eventually pain sensations. From the efferent autonomic nerve system, acetylcholine and norepinephrine are liberated in the nasal epithelium, together with the neuropeptides vasointestinal peptide (VIP) and neuropeptide Y (NPY). The pungent component of red pepper, capsaicin, is able to desensitize and eventually deplete the sensory nerve endings from neuropeptides like SP, CGRP, and NKA [70]. When capsaicin is applied to the nasal mucosa of patients with non-allergic rhinitis [78,89,141,152], they experience nasal complaints for a short while. Hereafter, they are more or less symptom-free for several weeks or months. This may demonstrate the role these neuropeptides play in causing nasal disorders. It is also known that a nonspecific hyperirritation of the nasal mucosa can occur in patients with allergic rhinitis [159]. Mediators of allergy are also capable of stimulating

sensory nerve endings [90,140]. Accordingly, the release of neuropeptides and the autonomic nerve reflexes caused through this may contribute to the hyperresponsiveness in allergic rhinitis. Indeed, nasal secretions of these patients show higher concentrations of neuropeptides [25,99,172].

The exact influence exerted by mediators of allergy and neuropeptides on mucociliary function is still under discussion in the literature. Their role remains further to be defined.

MEDIATORS OF ALLERGY, NEUROPEPTIDES AND MUCOCILIARY FUNCTION

In a type I IgE-mediated allergic reaction, as found in allergic rhinitis, several mediators are released, mainly from mast cells and basophilic granulocytes. These mediators may be preformed and stored in intracellular granula. Otherwise, they are newly formed in the allergic reaction, in most cases as a result of the arachidonic-acid metabolism.

Preformed mediators

Histamine is the best-known example of a preformed mediator. Chapter 4 reports on a study of how histamine affects ciliary activity. TAME-esterase (tosylarginine methyl ester esterase) represents a mixture of kinin-forming enzymes. It may be elevated in nasal secretions after nasal allergen challenge [102,103]. One of the kinins, bradykinin, is able to stimulate ciliary activity in vivo as well as in vitro [82,155]. In vitro, this effect was elicited by prostaglandin E_2 (PGE_2). However, this result could not be confirmed in vivo. Our experiments with PGE_2 are described in Chapter 5. At present, it is not known if other preformed substances have a distinct influence on mucociliary function. Examples of these compounds are chemotactic factors like ECF-A and HMW-NCF (eosinophil chemotactic factor of anaphylaxis and high-molecular weight neutrophil chemotactic factor), neutral proteases, exoglycosidases and proteoglycans.

Newly formed mediators

In the course of an allergic reaction, cell-membrane phospholipid is transformed enzymatically to arachidonic acid, which is further metabolized by cyclo-oxygenase or lipoxygenase.

The products of the cyclo-oxygenase pathway are the prostaglandins (PGs) PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane A_2 (TxA_2). Chapter 5 presents our findings and those of others on the effects of PGD_2 and PGE_2 on ciliary activity. $PGF_{2\alpha}$ is able to stimulate mucociliary activity in vivo in the rabbit

maxillary sinus [32]. However, no effect is found in vitro in ciliated cells from the trachea of sheep [173]. Besides, it is not certain to what extent $PGF_{2\alpha}$ contributes to nasal symptoms in humans; no increased levels have been measured after allergen challenge in allergic rhinitis patients [20]. PGI_2 rapidly degrades to the stable metabolite 6-keto $PGF_{1\alpha}$, which is pharmacologically inactive [162] and does not show increased levels after nasal allergen challenge [20]. However, the PGI_2 analog Iloprost is capable of letting CBF increase in human adenoid cilia in vitro [18]. Also TxA_2 is rapidly metabolized into the stable though inactive TxB_2. TxB_2 levels are found to be increased in nasal secretions after allergen provocation in patients suffering from nasal allergy [20]. The TxA_2 analog U46619 shows no effect on in vitro CBF [18].

The lipoxygenase pathway yields substances like the leukotrienes (LTs) LTB_4, LTC_4, and the group of HETEs (hydroxyeicosatetraenoic acids). LTC_4 is metabolized to LTD_4 and this, in turn, is metabolized into LTE_4. Together, these 3 LTs comprise SRS-A (slow-reactive substance of anaphylaxis). Chapter 4 presents our findings and discusses the influence of LTC_4 on ciliary activity. LTB_4 showed no effect on CBF of human nasal cilia [17]. The infuences of HETEs on mucociliary activity are not known.

Platelet activating factor (PAF, also known as PAF-acether or AGECP, alkylglyceryletherphosphorylcholine) is a membrane-derived mediator. It is not produced by the arachidonic acid metabolism [55]. PAF can decrease the CBF of human nasal and paranasal ciliated cells in vitro [44,45]. There is evidence of a direct toxic effect on ciliated cells [44], but there is also evidence of a receptor-mediated influence [45]. PAF is found to be elevated in nasal secretions after allergen challenge in allergic rhinitis [97]. Nevertheless, its role in the pathogenesis of allergic rhinitis is still a matter of discussion [6,14].

Neuropeptides

Many neuropeptides have been found in the airways [15,16]. Neuropeptides like SP, CGRP, and NKA can be released from trigeminal nerve endings. The effects of SP and CGRP on ciliary activity are described in Chapter 6 and 7 respectively. NKA can stimulate mucociliary activity in the rabbit maxillary sinus in vivo [79]. In vitro CBF rises in ciliated cells derived from rabbit tracheas by NKA [77], but it remains unchanged in those cells from guinea pig tracheas [75]. NPY is released together with norepinephrine from sympathetic nerves. NPY reduces mucociliary activity in vivo in the rabbit maxillary sinus model [24]; in vitro data are not available. However, the role of the sympathetic nerve system in nasal physiology and pathophysiology seems to be restricted [147]. VIP, which is liberated from parasympathetic

together with acetylcholine, does not affect mucociliary activity in the same rabbit experimental set-up [81]; in vitro data are lacking at present.

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CHAPTER 3

CELL SUSPENSION CULTURES AND ADENOID EPITHELIUM: AN ASSESSMENT OF THE SOURCE OF MATERIAL FOR HUMAN CILIARY FUNCTION EXPERIMENTS IN VITRO

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SUMMARY

The aim of this study was to explore the usefulness of 2 different in vitro models for studying the function of human upper respiratory cilia, i.e. cell suspension cultures of human upper airway epithelium, and ciliated adenoid epithelium. Ciliary beat frequency (CBF) and signal consistency (SC), as parameters of ciliary function, were measured by a computerized photo-electrical method. Measurements after 1 week revealed that CBF of ciliated aggregates from cell suspension cultures had deteriorated to a mean of 5.8 Hz. In the subsequent period, it remained at this rather low and non-physiological level. SC decreased too, although not as dramatically. These results indicate that ciliated aggregates from cell suspension cultures cannot be used for human ciliary function experiments in vitro. On the other hand, in ciliated adenoid epithelium, CBF remained constant for a period of 5 hours, although SC decreased after 30 min. Because of this CBF result and the fact that ciliated adenoid epithelium is easily obtainable, we regard this material as suitable for studying human ciliary beat in vitro.

KEY WORDS

Human cilia - cell suspension cultures - adenoid epithelium - ciliary beat frequency - ciliary beat harmony

INTRODUCTION

The mucociliary transport system is an important defense mechanism of the airways. A major factor in sustaining mucociliary transport is ciliary beat. Several methods for testing ciliary function have been described. Both animal and human ciliated epithelium have been used, taken from the upper and lower respiratory tract. For pharmacological studies the use of human specimens has to be preferred, because there is an interspecies variation in ciliary reaction [16].

Human specimens can be obtained from the lower airways and the nasal mucosa, the latter being the more accessible location. Both brushing [1,4] and curette techniques [5] yield ciliated epithelium without a proper lining, since many intercellular connnections are mechanically disrupted during harvesting. Mucosal biopsies provide much better specimens, as they have an intact lining of ciliated cells [5]. However, the biopsy method has certain practical disadvantages. Firstly, local anaesthetics cannot be used in view of their

ciliotoxic effects [6]. Secondly, there is a risk of nasal bleeding after biopsy. Alternatively, mucosa can be harvested from patients undergoing nasal or sinus surgery [18], but an important drawback of this method is that the mucosa is usually in a pathological condition. To circumvent the above-mentioned disadvantages, ciliated epithelium, obtained from patients undergoing adenoidectomy, can be used [9,15,16].

Furthermore, it was suggested that preserved or cultured respiratory epithelium can be used. Proetz and Pfingsten processed explants of animal ciliated epithelium [11,12], whereas Rose et al. used human nasal epithelium [14]. In an explant, the specimens are preserved under optimal physiological conditions. However, mitosis of ciliated cells does not take place [2]. Mitosis is not affected in a monolayer culture of dissociated cells, but the specific properties of the respiratory epithelium, i.e. the cilia and the mucus-producing cells, are lost in these cultures [13,19].

Jorissen et al. recently succeeded in preserving nasal epithelial cells with beating cilia in cell suspension cultures, which could be maintained for up to 7 months [8]. The dissociated cells formed aggregates of 50 µm to 2 mm in diameter, and with the cilia directed outwards. However, ciliary activity was not quantitatively determined.

Sufficient amounts of viable ciliated cells, in a functional state comparable to the physiological condition, are a prerequisite for performing in vitro studies on human ciliary function. The present study was designed to explore the usefulness of 2 different sources of human upper respiratory epithelium for in vitro function experiments: (1) ciliated aggregates taken from cell suspension cultures, and (2) ciliated epithelium of freshly harvested adenoids. Cell suspension cultures have the advantage of being at the researcher's disposal at any moment. An argument in favour of using adenoid epithelium is its ample availability in ENT practice.

Ciliary beat frequency (CBF) and signal consistency (SC) were used as parameters of ciliary activity. CBF is assumed to be a decisive factor in mucociliary transport [3], whereas SC may give an indication of ciliary harmony [7].

MATERIALS AND METHODS

Preparation of cell suspension cultures

Cell suspension cultures were prepared from the epithelium of nasal polyps (n=16) and the mucosa of inferior turbinates (n=7). Tissues were obtained from patients undergoing polypectomy or turbinate surgery under general anaesthesia. The specimens were processed according to the method described

by Jorissen et al. [8]. First, they were rinsed 3 times in medium consisting of Ham's F12-DME 1/1 (Gibco, Paisley, UK), NU serum (10%), penicillin G (50 IU/ml) and streptomycin (50 µg/ml). The tissues were then digested in 0.1% pronase (Sigma, St. Louis, MO, USA) at 4 °C under continuous rotation over a period of 24 hours. Next, the suspension was washed 3 times with medium and preplated in plastic culture dishes for one hour at 37 °C to remove fibroblasts. Cell suspensions were then placed on a shaker at 80 rpm for 7 days at 37 °C, enabling the formation of ciliated aggregates. They were stored under 5% CO_2 in an incubator. The medium was changed after the first day and subsequently 3 times a week.

Adenoid specimens

Adenoids were obtained from children undergoing adenoidectomy (n=7). For nearly all of them it was unknown whether they had an allergic constitution. Therefore, selecting adenoid specimens on that basis was not feasible. In order to remove blood and other debris, the specimens were rinsed in physiological saline shortly after adenoidectomy. They were then transferred to medium, which consisted of CMRL-1066 (Gibco, Paisley, UK) containing glutamine, 5% inactivated fetal calf serum, hydrocortisone hemisuccinate (0.1 µg/ml), crystalline porcine insulin (1 µg/ml), penicillin G (100 IU/ml) and streptomycin (100 µg/ml) [5,20]. Pieces of approximately 0.3 cm in diameter were cut from the ciliated epithelium and examined microscopically. Specimens were only used for measurement when a proper row of beating cilia was present.

Measurement of CBF and SC

CBF and SC were measured by the photoelectrical method, as described by Ingels et al. [5]. The specimens were inserted into a perfusion chamber, which consists of a standard glass slide and a cover slip with a silicone ring in between, mounted in an aluminium frame. The perfusion chamber was placed onto a microscope stage that was kept at a temperature of 34 °C by means of an electronic heating device. A phase-contrast microscope (Leitz, Wetzlar, Germany) was adapted by attaching a triocular tube, using a x100 oil-immersion objective and a x10 ocular lens. A square-angled diaphragm was mounted in the same tube with a beam splitter and a visaflex house (Leitz) in order to view the measured area. A 12 V/250 W halogen lamp was used as light source. Variations in light intensity, caused by the beating action of the cilia, were registered by a photometer. Using a 12-bit A/D converter, the digitalized signal was recorded by a personal computer (IBM, Grenock, UK) with a sample frequency of 200 Hz. A fast Fourier transform analysis (FFT) of the recorded signal was performed over a period of 20 sec. CBF

was determined from the first harmonic of the power spectrum obtained by FFT. On the basis of the CBF signal, the SC was computed.

Experimental design

The cell suspension cultures were examined daily in order to study the survival of ciliary activity over time. Furthermore, ciliary activity was determined by measuring CBF and SC at the outset, and at weekly intervals up to 7 weeks. Because the process of forming aggregates took a week, the first measurements could only be made after 1 week. To avoid microbial contamination, aggregates were not returned to their original cell suspension culture after measurement.

Adenoid specimens were investigated by measuring CBF and SC at the start and subsequently every 30 minutes for 5 hours. Since CBF of different cells in the same specimen can vary considerably [5], all measurements in each experiment were performed on one single ciliated cell. Only cells adjacent to others and beating freely were examined. Statistical analysis of the results was carried out by analysis of variance (ANOVA). A p value < 0.05 was considered significant.

RESULTS

Cell suspension cultures

The number of aggregates with beating cilia was found to diminish with time (Table 1). The mean functional survival time amounted to 19.7 days (range 3-50 days) for cell suspension cultures derived from polyp epithelium, and 9.4 days (range 3-22 days) for those from turbinate mucosa.

In Figure 1, CBF and SC are presented graphically in relation to time. The mean initial value of CBF (± SEM) was 8.8 Hz (± 0.2). After one week a mean value (± SEM) of 5.8 Hz (± 0.2) was found. In the subsequent period, CBF stayed at a relatively low and varying level, although some increase was observed after 5 weeks. SC, with a mean initial value (± SEM) of 2.3 (± 0.1), also showed lower values with time, although less dramatically than CBF.

Adenoid specimens

The results obtained in adenoid specimens are presented in Figures 2 and 3. CBF amounted to 9.3 Hz initially, and it remained at a constant level during the complete test period of 5 hours. Statistical analysis showed no significant CBF change over time (ANOVA, $p > 0.05$). For SC, a statistically significant time-dependent decrease was found (ANOVA, $p < 0.05$). After 90 minutes SC was about 60% of its initial value, and no further decrease was observed.

Table 1 Human polyp and turbinate epithelium in cell suspension cultures; survival of viable cells with beating cilia (mean time and range).

	number of cultures	time (days) mean ± SEM	range
Polyps	16	19.7 ± 3.9	3 - 50
Turbinates	7	9.4 ± 2.5	3 - 22

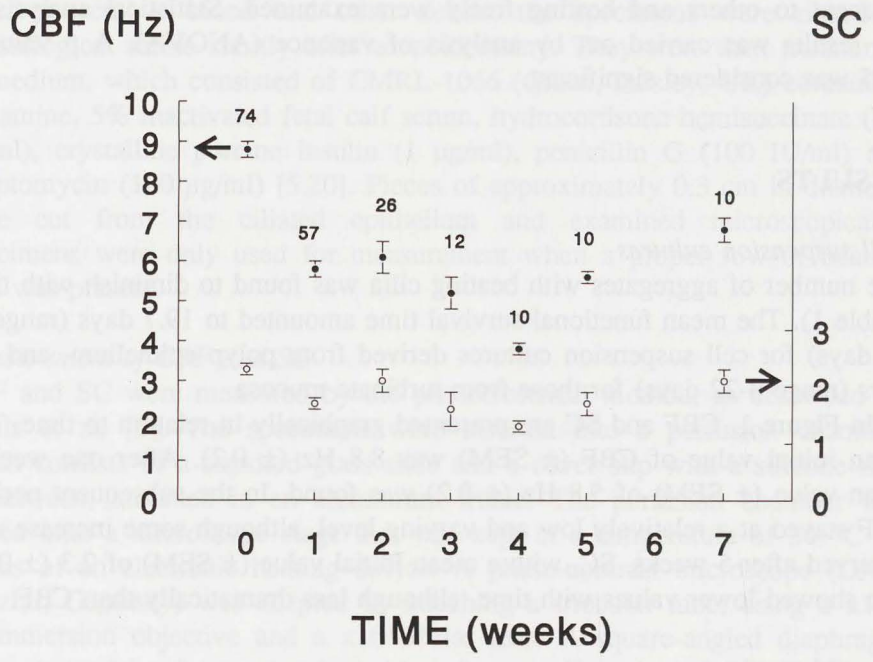

Figure 1 CBF (mean ± SEM; closed circles) and SC (mean ± SEM; open circles) of aggregates of cell suspension cultures. The number of measured ciliated cells is indicated.

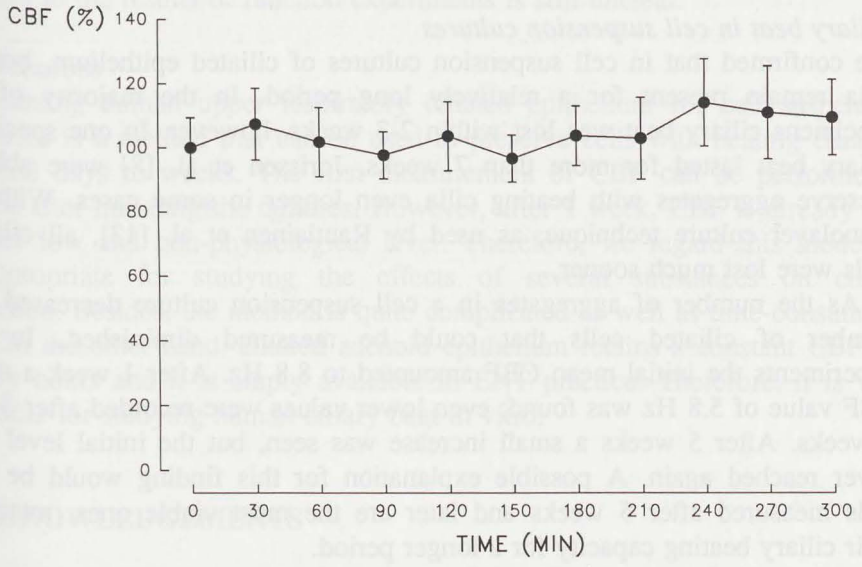

Figure 2 CBF (mean ± SEM; n=7) of adenoid cilia in relation to time. Initial mean CBF (9.3 Hz) is set at 100%.

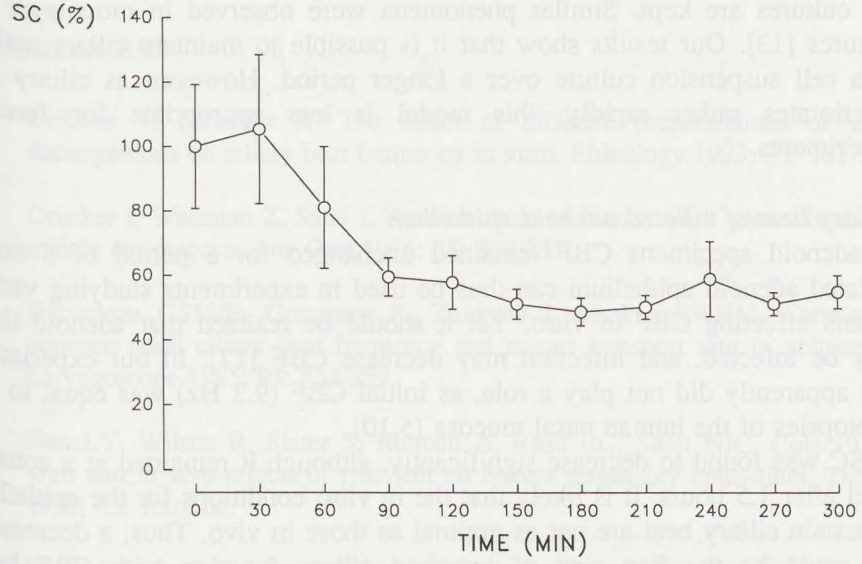

Figure 3 SC (mean ± SEM; n=7) of adenoid cilia in relation to time. Initial mean SC (3.0) is set at 100%.

DISCUSSION

Ciliary beat in cell suspension cultures

We confirmed that in cell suspension cultures of ciliated epithelium, beating cilia remain present for a relatively long period. In the majority of the specimens ciliary beat was lost within 2-3 weeks, however. In one specimen ciliary beat lasted for more than 7 weeks. Jorissen et al. [8] were able to preserve aggregates with beating cilia even longer in some cases. With the monolayer culture technique, as used by Rautiainen et al. [13], all ciliated cells were lost much sooner.

As the number of aggregates in a cell suspension culture decreased, the number of ciliated cells that could be measured diminished. In our experiments the initial mean CBF amounted to 8.8 Hz. After 1 week a mean CBF value of 5.8 Hz was found; even lower values were recorded after 3 and 4 weeks. After 5 weeks a small increase was seen, but the initial level was never reached again. A possible explanation for this finding would be that cells measured after 5 weeks and later are the most viable ones, retaining their ciliary beating capacity for a longer period.

Also, a minor decrease in SC was found in the course of time. The relevance of this parameter is not yet fully understood, but this decrease in SC could indicate a loss of co-ordination of the beating of the cilia. Apparently, ciliary beat becomes slower as well as less harmonic the longer the cultures are kept. Similar phenomena were observed in monolayer cell cultures [13]. Our results show that it is possible to maintain ciliary activity in a cell suspension culture over a longer period. However, as ciliary beat deteriorates rather rapidly, this model is less appropriate for function experiments.

Ciliary beat of ciliated adenoid epithelium

In adenoid specimens CBF remained unchanged for a period of 5 hours. Ciliated adenoid epithelium can thus be used in experiments studying various factors affecting CBF in vitro. Yet it should be realized that adenoid tissue may be infected, and infection may decrease CBF [17]. In our experiments this apparently did not play a role, as initial CBF (9.3 Hz) was equal to that in biopsies of the human nasal mucosa [5,10].

SC was found to decrease significantly, although it remained at a constant level after 1.5 hours. It is likely that the in vitro conditions for the epithelium to sustain ciliary beat are not as optimal as those in vivo. Thus, a decrease in SC could be the first sign of impaired ciliary function with CBF being unaffected. This finding supports the hypothesis that SC has a certain value

as another parameter of ciliary activity. However, the significance of SC with regard to the results of function experiments is still unclear.

Conclusions

Processing human upper respiratory ciliated epithelium for cell suspension cultures is a method that can be used to preserve cells with beating cilia for several days to weeks. The first measurement of CBF can be performed 1 week after initiating the cultures. However, after 1 week, CBF is already at a rather low and non-physiological level. Therefore, we regard this model as inappropriate for studying the effects of several substances on ciliary function. Besides, the method is quite complicated as well as time-consuming.

On the other hand, ciliated adenoid epithelium retains a constant CBF for many hours and it is amply available in ENT practice. Therefore, it is very suitable for studying human ciliary beat in vitro.

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CHAPTER 4

HISTAMINE AND LEUKOTRIENE C_4 EFFECTS ON IN VITRO CILIARY BEAT FREQUENCY OF HUMAN UPPER RESPIRATORY CILIA

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SUMMARY

Decreased mucociliary transport can occur in patients with type I (IgE-mediated) allergic rhinitis or allergic asthma. This study investigated if the mediators of allergy histamine and leukotriene C_4 (LTC_4) could interfere with ciliary beat frequency (CBF) of in vitro human upper respiratory cilia and eventually result in decreased mucociliary transport. Ciliated epithelium of human adenoid tissue was used in the experiments and CBF was determined using a computer-assisted photoelectrical method. Histamine in concentrations of 10^{-6}-10^{-3} M (n=12) and LTC_4 as 10^{-9}-10^{-6} M solutions (n=10) showed no statistically significant dose-dependent effect on CBF in vitro.

KEY WORDS

Allergic rhinitis - histamine - leukotriene C_4 - ciliary beat frequency - human upper respiratory cilia

INTRODUCTION

Mucociliary transport of the upper and lower airways is an important defense mechanism against inhaled particles, bacteria, and viruses [23]. However, mucociliary clearance may be impaired in patients with allergic rhinitis or allergic asthma [16,17]. This predisposes affected individuals to severe infections of the respiratory tract, as seen in patients with primary ciliary dyskinesia [22].

An important driving force in mucociliary transport is ciliary beat [7], so that mucociliary clearance can be decreased due to a decline in ciliary beat frequency (CBF). Indeed, in vitro measurements of biopsies from patients with nasal allergies reveal a low CBF [9,20]. Yet, alteration in mucus production or composition may also play a role in decreasing mucociliary clearance. Specifically, in type I (IgE-mediated) allergic reactions, as seen in patients with allergic rhinitis, several mediators of allergy are released from the nasal mucosa [3,19]. These mediators may in turn be responsible for the decline in CBF, which then impairs mucociliary clearance.

The aim of this study was to determine the effects of histamine and leukotriene C_4 (LTC_4) as mediators on the CBF of in vitro human upper respiratory cilia. Histamine, a potent mediator, is stored in the granules of mast cells and basophilic granulocytes [26]. Previous in vivo experiments utilizing the maxillary sinuses of rabbits demonstrated that an intra-arterial

injection of histamine could stimulate a rise in mucociliary activity up to 31.6% [6]. This effect was found to be blocked by an H_1-receptor antagonist.

According to clinical reports, tracheal and bronchial mucociliary clearance rises in asthmatic and non-asthmatic patients after histamine inhalation, occurring more sharply in asthmatic subjects [8,18]. Other investigators have found that histamine perfusion does not change the CBF of animal cilia in vitro [1,12,21] or shows stimulation at relatively high concentrations [24]. In human cilia, histamine perfusion shows no effect [2,14].

LTC_4 is a mediator that has been found in the lipooxygenase pathway in a type I allergic reaction [3]. In vivo mucociliary activity in the rabbit maxillary sinus is not changed by LTC_4 [5]. However, in vitro measurements show both depression and stimulation of the CBF of animal cilia [24,25], but only depression in human cilia [2].

In view of these conflicting results we studied the effects of histamine and LTC_4 on in vitro CBF of human upper respiratory cilia. Despite the preceding conflicting evidence, we were motivated to pursue this investigation by its clinical relevance to patients with nasal allergy. An in vitro system was used specifically to exclude the possible influence of mucus production.

MATERIALS AND METHODS

Tissue preparation

Ciliated epithelium from human adenoids was used in the experiments. This tissue was chosen because of its comparability in structure with other parts of the upper respiratory tract, as confirmed in a previous histological study [27]. The adenoids were obtained following routine adenoidectomies in children and were rinsed thoroughly in 0.9% saline solution to remove blood. Specimens were then transferred to CMRL-1066 medium (Gibco, Paisley, UK) containing glutamine, to which had been added 5% inactivated fetal calf serum, hydrocortisone hemisuccinate (0.1 µg/ml), crystalline porcine insulin (1 µg/ml), ß-retinyl acetate (0.1 µg/ml), penicillin G (100 U/ml) and streptomycin (100 µg/ml) [11,28]. Tissue pieces of approximately 0.3 cm in diameter were cut from specimens and were examined microscopically. When a proper row of beating cilia was found, the tissue piece was transferred to a perfusion chamber for measurement.

CBF measurements

A photoelectrical method, as described by Ingels et al. [11], was used for CBF measurements. Ciliated specimens were studied in a perfusion chamber, which consisted of a standard glass slide and cover glass separated by a

silicone ring and mounted in an aluminium frame. The perfusion chamber was placed onto a microscope stage, where the temperature was kept at 34 °C by means of an electronic heating device.

A phase-contrast microscope (Leitz, Wetzlar, Germany) was adapted by attaching a triocular tube, a x100 oil-immersion objective and a x10 ocular. A square-angled diaphragm was mounted in the tube with a beam-splitter and a visaflex house (Leitz) in order to view the measured area. A 12 V/250 W halogen lamp was used as light source. Variations in light intensity caused by the beating action of the cilia were detected by a photometer. Using a 12-bit A/D converter, the digitalized signal was recorded by a personal computer (IBM, Grenock, UK) with a sample frequency of 200 Hz.

A fast Fourier transform analysis (FFT) of the recorded signal was performed over a period of 20 s. The CBF was determined from the first harmonic of the power spectrum obtained by the FFT [11]. All CBF measurements in one experiment were performed on a single ciliated cell (about 200 cilia), since CBF varied between different cells in the same biopsy [10]. Only ciliated cells were examined that were adjacent to other cells and were able to make ciliary movements in a free space.

Experimental design

The initial CBF in all experiments was determined after perfusion with neutral medium. In order to exclude mechanical influences by perfusion, measurements of CBF were carried out 5 min after stopping the perfusion [10]. Each perfusion took place at a speed of 4 ml per 6 min, since no effect on CBF was found at this rate [10]. Since the volume of the perfusion chamber was 0.4 ml, contents of the chamber were replaced 10 times every 6 min. Either histamine dihydrochloride (Sigma, St. Louis, MO, USA) or LTC_4 (provided by the Department of Pharmacology, Utrecht University, The Netherlands) was added to the culture medium. The pH of the culture medium was 7.4 and remained constant because of the buffer capacity of the medium. After measuring the initial CBF, the chamber was perfused with increasing concentrations of either histamine (from 10^{-6} M to 10^{-3} M) or LTC_4 (10^{-9} M to 10^{-6} M). In the histamine experiments (n=12), CBF was recorded 5 min after each perfusion. In the LTC_4 experiments (n=10), recordings were made after 5 and 10 min in order to ascertain a possible effect of LTC_4 occurring at a later time. To determine if the effect of histamine or LTC_4 was reversible, the chamber was washed out with neutral medium after each perfusion with histamine or LTC_4. Subsequently, CBF was recorded once again 5 min after discontinuation of perfusion with culture medium.

Statistical analysis

All values are expressed as the mean ± SEM. Statistical analysis was carried out by analysis of variance (ANOVA). A p value < 0.05 was considered significant.

RESULTS

Histamine

The effect of perfusion of histamine on the mean CBF is represented in Table 1 and in Figure 1. The mean initial CBF was 9.9 ± 0.7 Hz and was set at 100% in Figure 1. Statistical analysis showed no significant dose-dependent effect of histamine.

LTC_4

The results for LTC_4 are represented in Table 2 and Figures 2 and 3. The mean initial CBF of 9.0 ± 0.3 Hz was set at 100%. Here too, no statistically significant dose-dependent effect was found.

Table 1 Effect of histamine on ciliary beat frequency (CBF) (mean ± SEM; n=12) 5 min after discontinuation of histamine perfusion.

Concentration (M)	CBF ± SEM (Hz)
Initial	9.9 ± 0.7
10^{-6}	10.1 ± 0.7
10^{-5}	9.7 ± 0.9
3.10^{-5}	9.3 ± 0.6
10^{-4}	9.0 ± 0.7
3.10^{-4}	9.5 ± 0.5
10^{-3}	10.2 ± 0.6

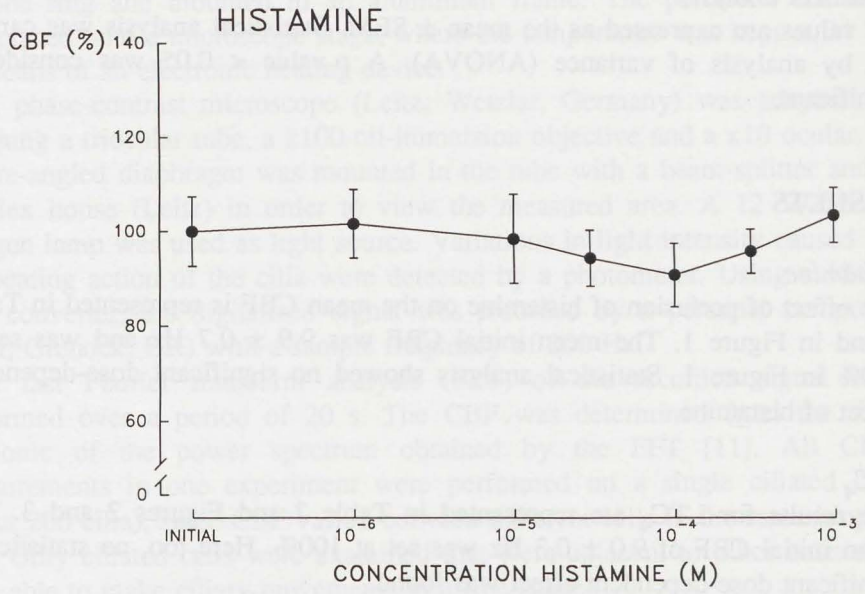

Figure 1 Effect of histamine on mean ciliary beat frequency (CBF) ± SEM (n=12). Initial mean CBF (9.9 Hz) is set at 100%.

Table 2 Effect of LTC_4 on CBF (mean ± SEM; n=10). CBF measurements 5 and 10 min after discontinuation of LTC_4 perfusion.

Concentration (M)	CBF ± SEM (Hz) t=5min	CBF ± SEM (Hz) t=10min
Initial	9.0 ± 0.3	
10^{-9}	9.0 ± 0.5	8.7 ± 0.4
10^{-8}	8.3 ± 0.3	8.1 ± 0.4
10^{-7}	8.7 ± 0.4	9.2 ± 0.4
10^{-6}	8.7 ± 0.4	8.3 ± 0.2

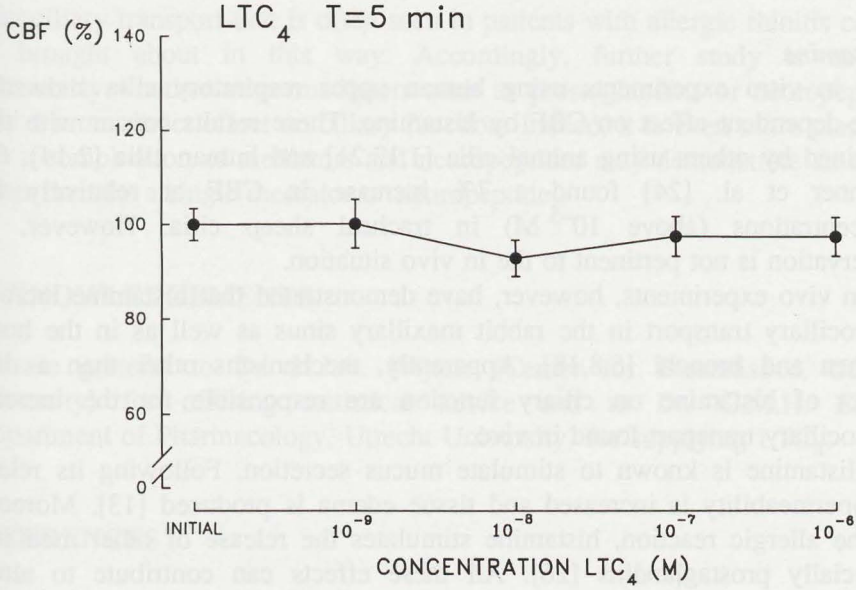

Figure 2 Effect of LTC_4 on mean CBF ± SEM (n=10) 5 min after discontinuation of perfusion. Initial CBF (9.0 Hz) is set at 100%.

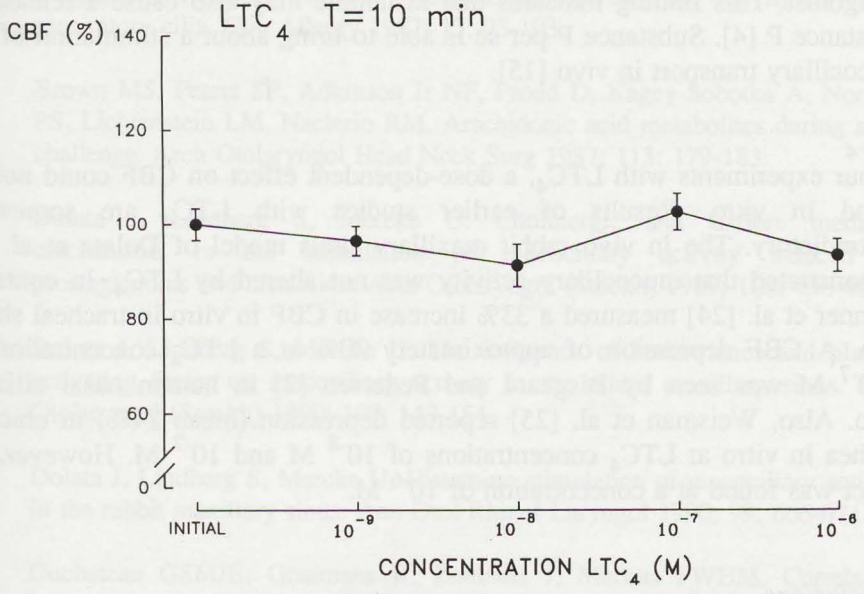

Figure 3 Effect of LTC_4 on mean CBF ± SEM (n=10) 10 min after discontinuation of perfusion. Initial CBF (9.0 Hz) is set at 100%.

DISCUSSION

Histamine

Our in vitro experiments using human upper respiratory cilia showed no dose-dependent effect on CBF by histamine. These results concur with those obtained by others using animal cilia [1,12,21] and human cilia [2,14]. Only Wanner et al. [24] found a 7% increase in CBF at relatively high concentrations (above 10^{-5} M) in tracheal sheep cilia. However, that observation is not pertinent to the in vivo situation.

In vivo experiments, however, have demonstrated that histamine increases mucociliary transport in the rabbit maxillary sinus as well as in the human trachea and bronchi [6,8,18]. Apparently, mechanisms other than a direct effect of histamine on ciliary function are responsible for the increased mucociliary transport found in vivo.

Histamine is known to stimulate mucus secretion. Following its release, vasopermeability is increased and tissue edema is produced [13]. Moreover, in the allergic reaction, histamine stimulates the release of other mediators, especially prostaglandins [26]. All these effects can contribute to altered mucociliary transport.

The stimulation of mucociliary transport in the rabbit maxillary sinus by histamine in vivo has been reported to be reduced by a substance-P antagonist. This finding indicates that histamine may also cause a release of substance P [4]. Substance P per se is able to bring about a stimulation of the mucociliary transport in vivo [15].

LTC_4

In our experiments with LTC_4, a dose-dependent effect on CBF could not be found in vitro. Results of earlier studies with LTC_4 are somewhat contradictory. The in vivo rabbit maxillary sinus model of Dolata et al. [5] demonstrated that mucociliary activity was not altered by LTC_4. In contrast, Wanner et al. [24] measured a 33% increase in CBF in vitro in tracheal sheep cilia. A CBF depression of approximately 20% at a LTC_4 concentration of 3.10^{-7} M was seen by Bisgaard and Pedersen [2] in human nasal cilia in vitro. Also, Weisman et al. [25] reported depression (mean 24%) in chicken trachea in vitro at LTC_4 concentrations of 10^{-8} M and 10^{-7} M. However, no effect was found at a concentration of 10^{-6} M.

Conclusions

Neither histamine nor LTC_4 were found to affect the CBF of human upper respiratory cilia in vitro in our experiments. We conclude that these mediators

have no direct effect on ciliary activity. Therefore, the decrease in mucociliary transport that is often seen in patients with allergic rhinitis cannot be brought about in this way. Accordingly, further study is needed, particularly whether other mediators such as prostaglandins or neuropeptides have a more direct effect on ciliary function. Indeed, it is even more plausible that a combination of mediators and neuropeptides may demonstrate an effect rather than by a single mediator or neuropeptide.

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CHAPTER 5

EFFECTS OF PROSTAGLANDINS D_2 AND E_2 ON CILIARY BEAT FREQUENCY OF HUMAN UPPER RESPIRATORY CILIA IN VITRO

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SUMMARY

Diminished mucociliary transport can occur in a type I (IgE-mediated) allergic reaction. We determined the effects of the mediators of allergy prostaglandin D_2 (PGD_2) and prostaglandin E_2 (PGE_2) on the ciliary beat frequency (CBF) of human upper respiratory cilia in vitro. Human adenoid tissue was used as the source for ciliated epithelium. CBF was measured by a computerized photoelectrical method. PGD_2 (10^{-8}-10^{-5} M, n=7) showed no statistically significant effect on CBF. PGE_2 (10^{-9}-10^{-6} M, n=10) caused a significant dose-dependent stimulation, with a maximum of 37% (ANOVA, $p < 0.001$). Therefore, prostaglandins D_2 and E_2 thus do not exert a direct negative influence on ciliary activity, which could account for a decrease in mucociliary transport. The stimulating effect of PGE_2 may be relevant in promoting mucociliary clearance in vivo.

KEY WORDS

Allergic rhinitis - prostaglandin D_2 - prostaglandin E_2 - ciliary beat frequency - human upper respiratory cilia

INTRODUCTION

Mucociliary clearance of the upper and lower airways can be depressed in allergic rhinitis and asthma [13,14]. In a type I (IgE-mediated) allergic reaction, as seen in nasal mucosa, many mediators of allergy are released [5,15]. These mediators could be responsible for diminished mucociliary transport by altering the mucus secretion or decreasing the ciliary activity. After nasal allergen challenge, ciliary beat frequency (CBF) was reported to be decreased in allergic subjects [8,17].

Of the mediators of allergy, several prostaglandins are newly formed arachidonic acid metabolites in the cyclooxygenase pathway. Higher levels of prostaglandin D_2 (PGD_2) and prostaglandin E_2 (PGE_2) were present in nasal washings of allergic subjects after challenge [5,15]. In contrast, levels of other prostaglandins, like prostaglandin $F_{2\alpha}$ and 6-keto-prostaglandin $F_{1\alpha}$, did not change after challenge. PGD_2 could only be demonstrated in the early allergic reaction [16], when mast cells play a dominant role. Furthermore, the concentration of this mediator was higher in patients with a perennial allergic rhinitis than in control subjects [11]. When applied to nasal mucosa, PGD_2 brought about a dose-related increase in nasal obstruction [7]. In this respect,

PGD_2 appeared to be 10 times more potent than histamine. An H_1-receptor antagonist could hardly attenuate this obstruction [9]. PGE_2 was reported to stimulate nasal vasoconstriction in pigs [3], leading to better nasal patency. Indeed, topically applied PGE_2 decreased nasal resistance in a majority of human volunteers [1], and in this respect it showed an opposite effect compared with PGD_2.

The aim of this study was to investigate the effects of both mediators on upper respiratory ciliary activity. Differences in ciliary reaction have been described for various species [21]. Therefore, human cilia is preferred in studies of this type. An in vitro system was used, in order to exclude the effects of mucus production.

MATERIALS AND METHODS

Tissue preparation

Ciliated epithelium of human adenoids, obtained by adenoidectomy, was used as previously described [18]. The adenoid tissue was transferred to the medium CMRL-1066 (Gibco, Paisley, UK), which contained several additives [24]. Pieces of approximately 0.3 cm were then cut from the ciliated epithelium. Specimens were only used for measurement when a proper row of beating cilia was found by microscopic inspection.

CBF measurements

A computerized photoelectrical method, as described by Ingels et al. [10], was used to measure CBF. The ciliated specimens were examined under a phase-contrast microscope (Leitz, Wetzlar, Germany) in a perfusion chamber. An electronic heating device kept the chamber at 34 °C. The beating action of the cilia caused the light intensity to vary. These variations were detected by a photometer and digitalized by an A/D converter. CBF was determined from the first harmonic of the power spectrum, obtained by fast Fourier transform analysis of the recorded signal. All CBF measurements in one experiment were performed on a single ciliated cell (about 200 cilia).

Experimental design

CBF was initially determined after perfusion with neutral medium. Then, PGD_2 (Sigma, St. Louis, MO, USA) or PGE_2 (Sigma, St. Louis, MO, USA) was added to the medium. This did not change the pH (7.4) because of the buffer capacity of the medium. Next, the chamber was perfused with increasing concentrations of either PGD_2 (10^{-8}-10^{-5} M, n=7) or PGE_2 (10^{-9}-10^{-6} M, n=10). CBF was recorded 5 and 10 min after perfusion was

discontinued. The chamber was washed out with neutral medium after each perfusion with PGD_2 or PGE_2 to determine if the effect was reversible. Subsequently, CBF was again recorded 5 min after stopping perfusion with neutral medium.

Statistical analysis

All values given here are means ± SEM. Statistical analysis consisted of analysis of variance (ANOVA), whereby a p value < 0.05 was considered significant.

RESULTS

PGD_2

The results for PGD_2 are shown in Table 1 and Figures 1 and 2. Statistical analysis did not reveal a significant dose-dependent effect on CBF either 5 min or 10 min after perfusion was discontinued. As expected, washing out with neutral medium had no effect (data not shown).

PGE_2

Table 2 and Figures 3 and 4 depict the results for PGE_2. A significant dose-dependent increase in CBF was found, both for the measurements after 5 min ($p < 0.001$) and 10 min ($p < 0.001$). At a concentration of 10^{-6} M, this increase reached a high 37.3 % and 33.7 %, respectively. No statistically significant difference was found between values after perfusion with PGE_2 and neutral medium was discontinued.

DISCUSSION

PGD_2

To our knowledge, this study is the first one to determine the effect of PGD_2 on human upper respiratory cilia. PGD_2 is a major prostaglandin released in the early phase of a type I allergic reaction [5,15,16]. Accordingly, it could have a direct negative effect on ciliary function. However, our experiments did not show such an effect in vitro. Furthermore, the role of PGD_2 in mucociliary transport in vivo has not been clarified yet. Experiments using selective blockers of PGD_2 [2] might elucidate its effects on mucociliary clearance.

Table 1 Effect of PGD_2 on CBF (mean ± SEM), n=7. CBF measurements made 5 and 10 min after discontinuation of PGD_2 perfusion.

Concentration (M)	CBF ± SEM (Hz) t=5min	CBF ± SEM (Hz) t=10min
Initial	10.6 ± 0.8	
10^{-8}	10.9 ± 1.2	11.2 ± 0.8
10^{-7}	10.6 ± 1.0	11.5 ± 1.3
10^{-6}	10.5 ± 1.3	11.1 ± 1.5
10^{-5}	10.1 ± 0.5	11.3 ± 0.9

Table 2 Effect of PGE_2 on CBF (mean ± SEM), n=10. CBF measurements made after discontinuation of perfusion with PGE_2 (5 and 10 min) and with neutral medium (5 min).

Concentration (M)	CBF ± SEM (Hz) t=5min	CBF ± SEM (Hz) t=10min	CBF ± SEM (Hz) after neutral medium
Initial	8.3 ± 0.6		
10^{-9}	8.0 ± 0.7	8.0 ± 0.5	8.9 ± 0.5
10^{-8}	8.5 ± 0.5	9.2 ± 0.6	9.0 ± 0.5
10^{-7}	9.8 ± 0.5	9.6 ± 0.5	9.8 ± 0.5
10^{-6}	11.4 ± 0.6	11.1 ± 0.6	10.1 ± 0.5

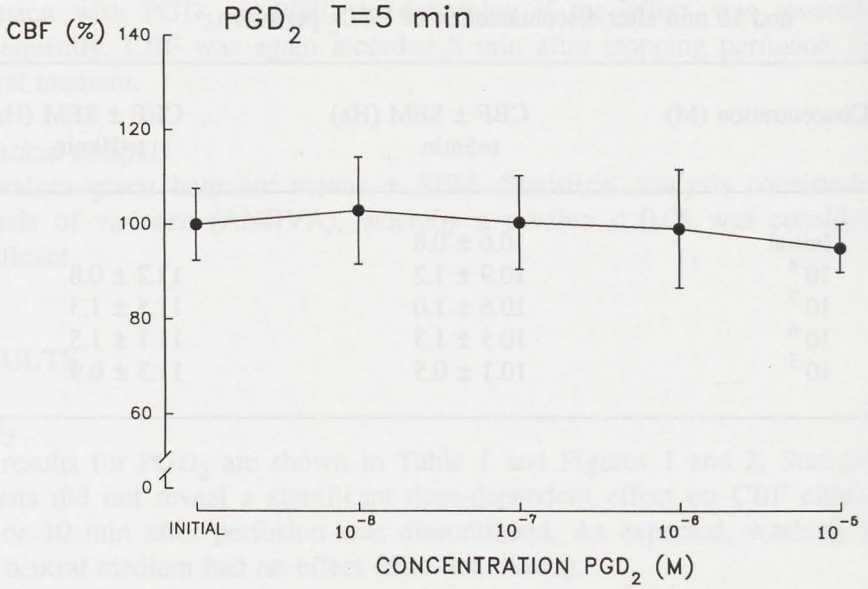

Figure 1 Effect of PGD_2 on mean CBF ± SEM (n=7), 5 min after discontinuation of perfusion. Initial CBF (10.6 Hz) is set at 100%.

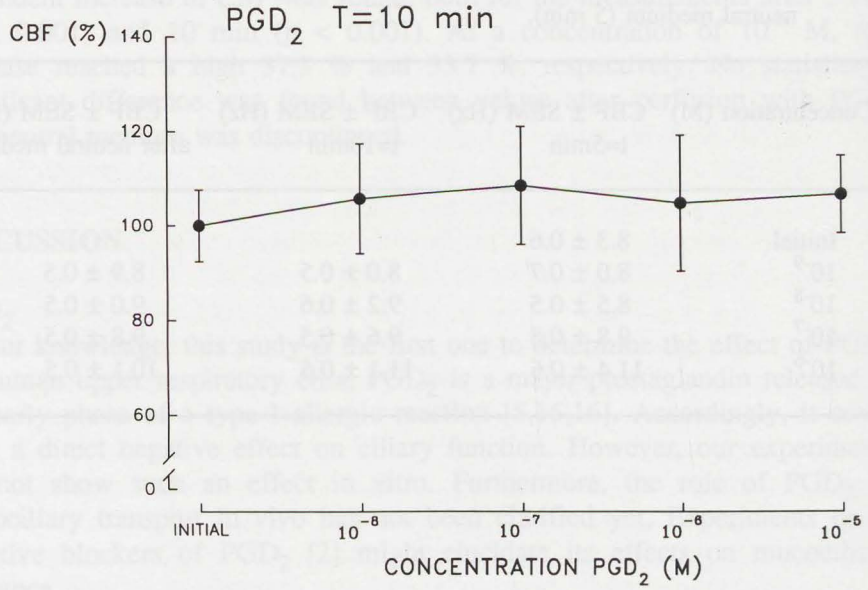

Figure 2 Effect of PGD_2 on mean CBF ± SEM (n=7), 10 min after discontinuation of perfusion. Initial CBF (10.6 Hz) is set at 100%.

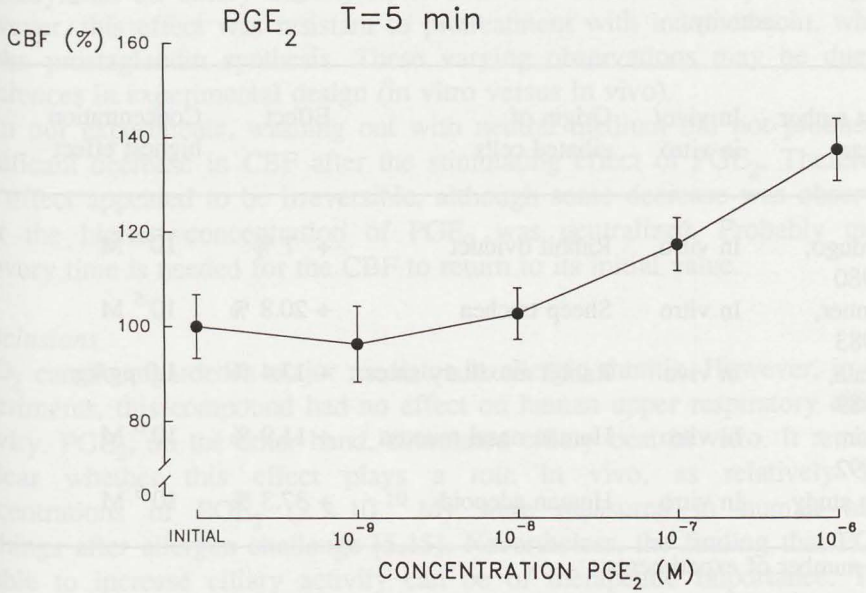

Figure 3 Effect of PGE_2 on mean CBF ± SEM (n=10), 5 min after discontinuation of perfusion. Initial CBF (8.3 Hz) is set at 100%.

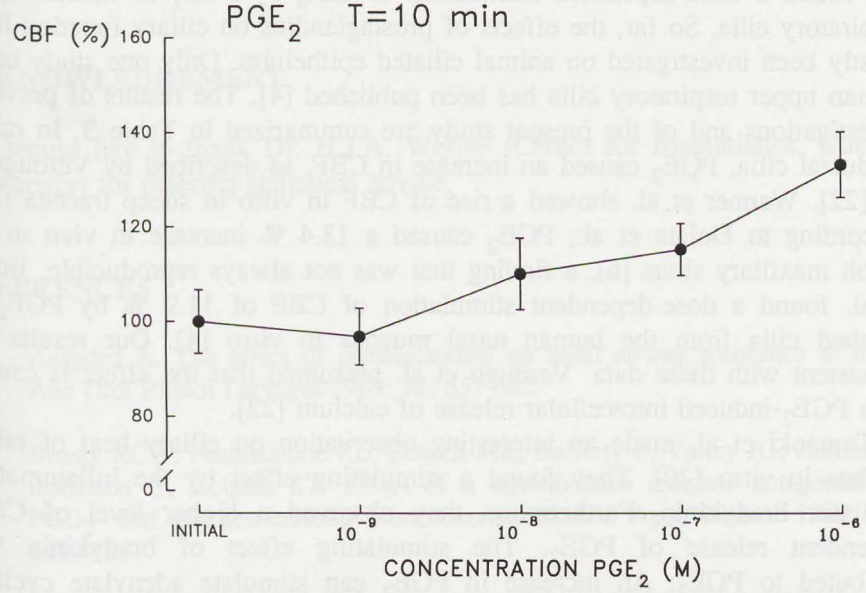

Figure 4 Effect of PGE_2 on mean CBF ± SEM (n=10), 10 min after discontinuation of perfusion. Initial CBF (8.3 Hz) is set at 100%.

Table 3 Observations of several authors concerning the effect of PGE_2 on ciliary activity.

First author, year	In vivo/ in vitro	Origin of ciliated cells	Effect	Concentration highest effect	n
Verdugo, 1980	In vitro	Rabbit oviduct	+ 7 %	10^{-6} M	8
Wanner, 1983	In vitro	Sheep trachea	+ 20.8 %	10^{-5} M	4
Dolata, 1989	In vivo	Rabbit maxillary sinus	+ 13.4 %	1.0 µg/kg	7
Bonin, 1992	In vitro	Human nasal mucosa	+ 11.9 %	10^{-6} M	7
This study	In vitro	Human adenoid	+ 37.3 %	10^{-6} M	10

n = number of experiments

PGE_2

We found a dose-dependent stimulation of CBF by PGE_2 in human upper respiratory cilia. So far, the effects of prostaglandins on ciliary function have mostly been investigated on animal ciliated epithelium. Only one study using human upper respiratory cilia has been published [4]. The results of previous investigations and of the present study are summarized in Table 3. In rabbit oviductal cilia, PGE_2 caused an increase in CBF, as described by Verdugo et al. [22]. Wanner et al. showed a rise of CBF in vitro in sheep trachea [23]. According to Dolata et al., PGE_2 caused a 13.4 % increase in vivo in the rabbit maxillary sinus [6], a finding that was not always reproducible. Bonin et al. found a dose-dependent stimulation of CBF of 11.9 % by PGE_2 in brushed cilia from the human nasal mucosa in vitro [4]. Our results are consistent with these data. Verdugo et al. presumed that the effect is caused by a PGE_2-induced intracellular release of calcium [22].

Tamaoki et al. made an interesting observation on ciliary beat of rabbit trachea in vitro [20]. They found a stimulating effect by the inflammatory mediator bradykinin. Furthermore, they observed a higher level of Ca^{2+}-dependent release of PGE_2. The stimulating effect of bradykinin was attributed to PGE_2. An increase in PGE_2 can stimulate adenylate cyclase. This, in turn, can raise the cyclic AMP level [19], which is an important factor in ciliary motility. Lindberg and Mercke also found a stimulating effect

of bradykinin on ciliary activity in vivo in the rabbit maxillary sinus [12]. However, this effect was resistant to pretreatment with indomethacin, which blocks prostaglandin synthesis. These varying observations may be due to differences in experimental design (in vitro versus in vivo).

In our experiments, washing out with neutral medium did not produce a significant decrease in CBF after the stimulating effect of PGE_2. Therefore, this effect appeared to be irreversible, although some decrease was observed after the highest concentration of PGE_2 was neutralized. Probably more recovery time is needed for the CBF to return to its initial value.

Conclusions

PGD_2 can be regarded a major mediator in allergic rhinitis. However, in our experiments, this compound had no effect on human upper respiratory ciliary activity. PGE_2, on the other hand, stimulated ciliary beat in vitro. It remains unclear whether this effect plays a role in vivo, as relatively low concentrations of PGE_2 (2×10^{-10} M) were measured in human nasal washings after allergen challenge [5,15]. Nevertheless, the finding that PGE_2 is able to increase ciliary activity can be of therapeutic importance. This insight may be useful in pathological states of the respiratory mucosa in which mucociliary clearance is depressed. More research is needed to assess the possible value of PGE_2 in stimulating mucociliary transport in vivo.

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CHAPTER 6

SUBSTANCE P AND CILIARY BEAT OF HUMAN UPPER RESPIRATORY CILIA IN VITRO

This chapter has been submitted for publication in
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SUMMARY

On stimulation of trigeminal nerve endings, neuropeptides are released into the nasal mucosa. Among these neuropeptides is substance P (SP). In this study, we determined the effect in vitro of SP as well as SP together with thiorphan, a blocker of the SP-degrading enzyme neutral endopeptidase, on the ciliary beat frequency (CBF) of the human upper respiratory tract. Ciliated epithelium of human adenoid tissue was used in the experiments. CBF was measured by means of a computer-assisted photoelectrical method. SP (10^{-8}-10^{-5} M, n=7) showed a small but statistically significant dose-dependent decrease in CBF. On perfusion with SP (10^{-8}-10^{-5} M, n=8) in combination with thiorphan, no statistically significant effect was found. We conclude that SP does not have a direct effect on ciliary activity to such an extent that it will affect mucociliary transport in vivo.

KEY WORDS

Substance P - thiorphan - ciliary beat frequency - human upper respiratory cilia

INTRODUCTION

Mucociliary transport is the first line of defense in the respiratory tract. The nose in particular is exposed to inhaled substances. In the nasal mucosa, neuropeptides like substance P (SP), calcitonin gene-related peptide (CGRP), and neurokinin A (NKA) have been demonstrated in trigeminal nerve endings [1-3,5,6,18,27]. In parasympathetic nerves, the neuropeptide vasointestinal peptide (VIP) coexists with acetylcholine, while sympathetic nerves contain neuropeptide Y together with norepinephrine [1-4,7].

On physical, thermal, or chemical stimulation of sensory trigeminal nerve endings, a reflex pathway is activated. This results in sneezing, itching, and sometimes in pain sensations. Acetylcholine with VIP and norepinephrine with neuropeptide Y are released from efferent autonomic nerves. Moreover, neuropeptides like SP, CGRP, and NKA are liberated into the nasal mucosa by a local axon reflex in otherwise afferent sensory nerve fibers [8]. Binding sites for neuropeptides have been demonstrated in the nasal epithelium [4-7]. Not only possible harmful effects can activate sensory nerve endings; also mediators of allergy are able to do so [19,24]. For example, increased levels of SP were found in nasal washings of patients with allergic rhinitis [9,20].

Exposure of the nasal mucosa to SP led to local symptoms with predominance of blockage [21], as well as to measurement of a rise in nasal resistance [10,29]. Furthermore, SP evoked atropine-resistant nasal secretion in animals [22]. However, this effect could not be demonstrated in man, possibly due to the rapid degradation of SP in human nasal secretions.

The aim of this study was to investigate the effect of SP on the ciliary beat frequency (CBF) of the upper respiratory tract. CBF is an important parameter of the activity of the mucociliary apparatus [11]. As species differences have been described in ciliary reaction patterns [28], we studied the effect of SP on human upper respiratory cilia. An in vitro system was used to exclude effects on ciliary beat caused by an altered amount or composition of mucus. SP is rapidly degraded enzymatically. Therefore, in a second series of experiments, we used thiorphan as a blocker of the SP-degrading enzyme neutral endopeptidase [23]. The latter is a peptide, bound to the cell membrane.

MATERIALS AND METHODS

Tissue preparation

We used the ciliated epithelium of human adenoids, obtained by adenoidectomy. The method of processing was described in detail in an earlier paper [25]. Briefly, the adenoids were first rinsed in 0.9 % saline solution and then transferred to the medium CMRL-1066 (Gibco, Paisley, UK) containing several additives [13,32]. Pieces of approximately 0.3 cm in diameter were cut from the ciliated epithelium. Next, the specimens were studied under the microscope in order to find a proper row of beating cilia. When such a row was found, the specimen was used for measurement.

CBF measurements

We used the computerized photoelectrical method for the CBF measurements, as described by Ingels et al. [13]. The ciliated specimens were brought into a perfusion chamber placed onto a phase-contrast microscope (Leitz, Wetzlar, Germany). Variations in light intensity were detected by a photometer and digitalized by an A/D converter. These variations were caused by the beating action of the cilia. Then, a power spectrum, obtained by fast Fourier transform analysis of the recorded signal, was computed. CBF was determined from the first harmonic of this power spectrum. All CBF measurements in one experiment were performed on a single ciliated cell (about 200 cilia). The temperature in the perfusion chamber was kept at 34 °C by means of an electronic heating device.

Experimental design

Either SP (Sigma, St. Louis, MO, USA) or SP together with thiorphan (Sigma, St. Louis, MO, USA) was added to the neutral medium. Because of the buffer capacity of the medium, no changes in pH (7.4) occurred. After perfusion with neutral medium, the initial CBF was determined. In the first series of experiments (n=7), the chamber was perfused with increasing concentrations of SP (10^{-8}-10^{-5} M). In the second series (n=8), SP (10^{-8}-10^{-5} M) was perfused with a constant amount of thiorphan (10^{-5} M). This concentration of thiorphan was chosen to bring about blockage of neutral endopeptidase [16]. CBF recordings were made 5 and 10 min after discontinuation of perfusion of each concentration. To determine if any effect was reversible, a wash-out procedure with neutral medium was carried out after each perfusion. Final CBF was recorded once again 5 min after stopping perfusion with neutral medium.

Statistical analysis

All values are expressed as the mean ± SEM. Statistical analysis was carried out by analysis of variance (ANOVA). A p value < 0.05 was considered significant.

RESULTS

Substance P

The results for SP are shown in Table 1 and in Figures 1 and 2. The mean initial CBF was 10.5 Hz; this level is set at 100% in Figure 1 and 2. After 5 min, the SP concentrations 10^{-8} and 10^{-7} showed a small increase in CBF: 1.9 % and 6.7 % respectively. Higher concentrations (10^{-6} and 10^{-5} M) demonstrated a decrease in CBF (13.3 % and 10.5 %), however. After 10 min, only depression of CBF was found. Overall, statistical analysis revealed a small but significant dose-dependent decrease in CBF, both 5 and 10 min after discontinuation of perfusion ($p < 0.01$ and $p < 0.05$ respectively). After washing out with neutral medium, no statistically significant effect could be found anymore (data not shown).

Substance P and thiorphan

Table 2 and Figures 3 and 4 present the results for SP together with thiorphan. The mean initial CBF amounted to 10.8 Hz. After 5 and 10 min, an increase in CBF was found at a SP concentration of 10^{-8} M: 4.6 % and 3.8 % respectively. Higher concentrations either had no effect or depressed CBF slightly. Statistical analysis revealed no significant dose-dependent effect by SP and thiorphan on CBF, neither after 5 min, nor after 10 min.

Table 1 Effect of SP on CBF (mean ± SEM), n=7. CBF measurements 5 and 10 min after discontinuation of perfusion.

Concentration SP (M)	CBF ± SEM (Hz) after 5 min	CBF ± SEM (Hz) after 10 min
Initial	10.5 ± 0.4	
10^{-8}	10.7 ± 0.2	10.0 ± 0.4
10^{-7}	11.2 ± 0.6	10.4 ± 0.4
10^{-6}	9.1 ± 0.3	9.2 ± 0.5
10^{-5}	9.4 ± 0.4	9.0 ± 0.3

Table 2 Effect of SP with thiorphan (10^{-5} M) on CBF (mean ± SEM), n=8. CBF measurements 5 and 10 min after discontinuation of perfusion.

Concentration SP (M)	CBF ± SEM (Hz) after 5 min	CBF ± SEM (Hz) after 10 min
Initial	10.8 ± 1.0	
10^{-8}	11.2 ± 1.1	11.3 ± 1.1
10^{-7}	10.3 ± 1.1	10.8 ± 1.2
10^{-6}	10.8 ± 1.1	10.5 ± 1.1
10^{-5}	10.7 ± 1.3	10.7 ± 1.0

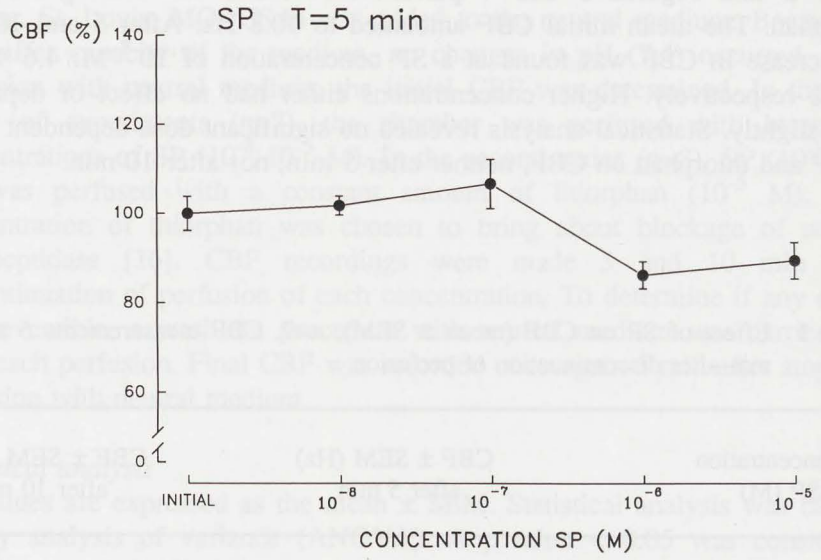

Figure 1 Effect of SP on mean CBF ± SEM (n=7), CBF measurements 5 min after discontinuation of perfusion. Initial CBF (10.5 Hz) is set at 100%.

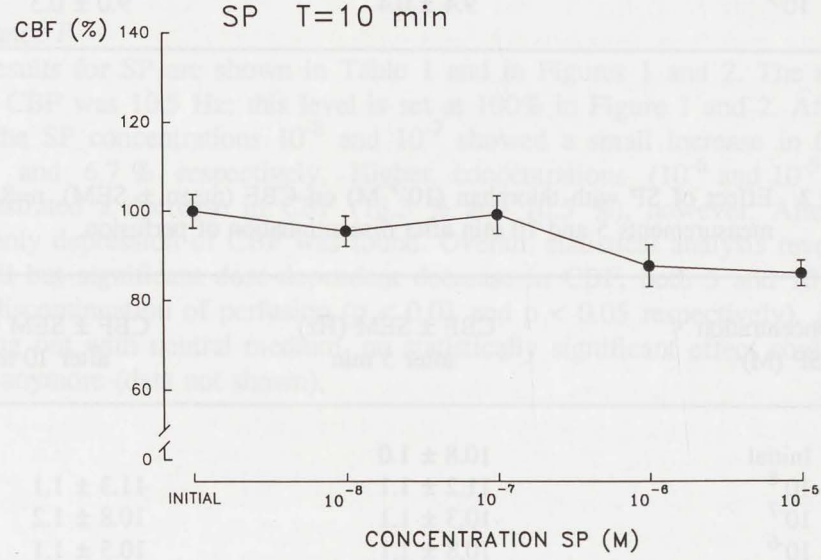

Figure 2 Effect of SP on mean CBF ± SEM (n=7), CBF measurements 10 min after discontinuation of perfusion. Initial CBF (10.5 Hz) is set at 100%.

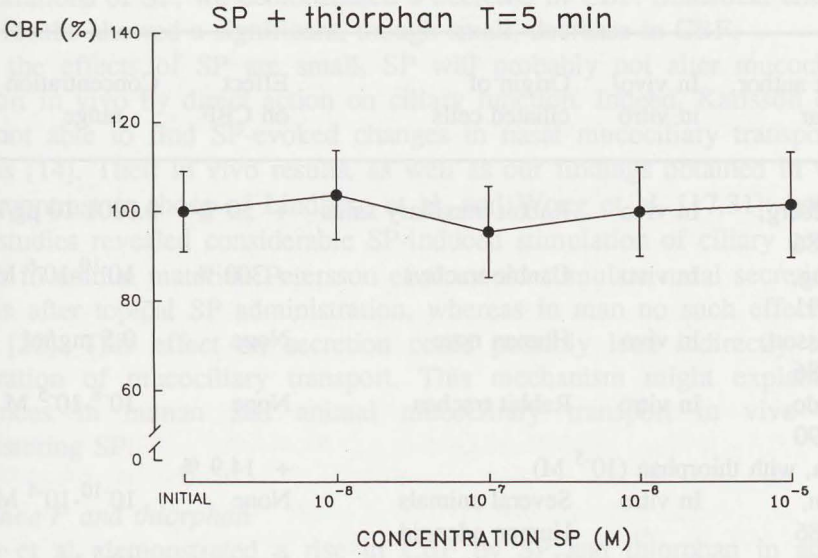

Figure 3 Mean CBF ± SEM (n=8) after administration of SP together with thiorphan (10^{-5} M), CBF measurements 5 min after discontinuation of perfusion. Initial CBF (10.8 Hz) is set at 100%.

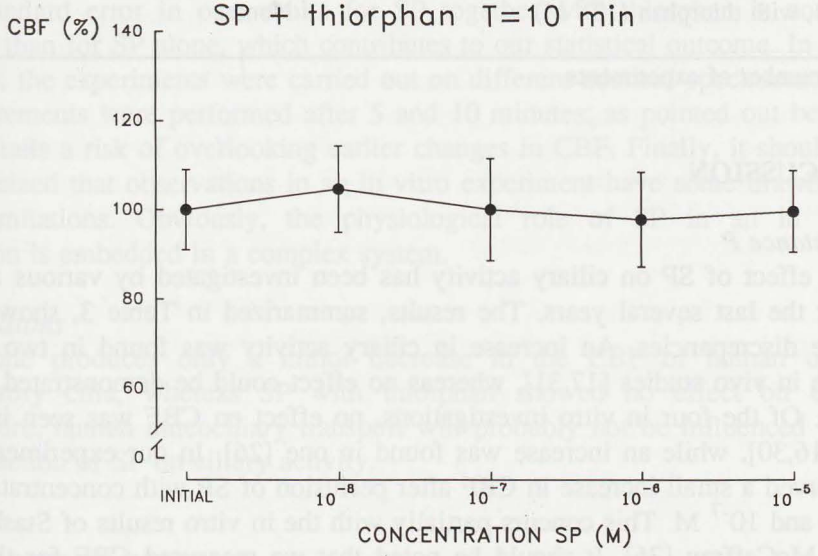

Figure 4 Mean CBF ± SEM (n=8) after administration of SP together with thiorphan (10^{-5} M), CBF measurements 10 min after discontinuation of perfusion. Initial CBF (10.8 Hz) is set at 100%.

Table 3 Effect of SP on ciliary activity as studied by several authors.

First author, year	In vivo/ in vitro	Origin of ciliated cells	Effect on CBF	Concentration range	n
Lindberg, 1986	In vivo	Rabbit maxillary sinus	+ 50 %	0.0001-10 µg/kg	14
Wong, 1991	In vivo	Canine trachea	+ 300 %	10^{-10}-10^{-6} M	8
Karlsson, 1986	In vivo	Human nose	None	0.5 mg/ml	13
Kondo, 1990	In vitro	Rabbit trachea	None	10^{-8}-10^{-5} M	5
idem, with thiorphan (10^{-5} M)			+ 14.9 %		8
Khan, 1986	In vitro	Several animals	None	10^{-10}-10^{-4} M	6-7
		Human adenoid			2-7
Wolf, 1988	In vitro	Human nose	None	10^{-5} M	2
Staskowski, 1992	In vitro	Human adenoid	+ 12.1 %	10^{-7}-10^{-4} M	10
This study	In vitro	Human adenoid	- 13.3 %	10^{-8}-10^{-5} M	7
idem, with thiorphan (10^{-5} M)			None		8

n = number of experiments

DISCUSSION

Substance P

The effect of SP on ciliary activity has been investigated by various authors over the last several years. The results, summarized in Table 3, show rather large discrepancies. An increase in ciliary activity was found in two of the three in vivo studies [17,31], whereas no effect could be demonstrated in one [14]. Of the four in vitro investigations, no effect on CBF was seen in three [15,16,30], while an increase was found in one [26]. In our experiments, we observed a small increase in CBF after perfusion of SP with concentrations of 10^{-8} and 10^{-7} M. This concurs partially with the in vitro results of Staskowski and McCaffrey [26]. It should be noted that we measured CBF for the first time 5 min after discontinuation of perfusion. Thus, an earlier effect caused by SP could have been overlooked. However, an early stimulating effect can also, at least partially, be a mechanical effect of perfusion [12]. For higher

concentrations of SP, we demonstrated a decrease in CBF. Statistical analysis of the results showed a significant, though small, decrease in CBF.

As the effects of SP are small, SP will probably not alter mucociliary transport in vivo by direct action on ciliary function. Indeed, Karlsson et al. were not able to find SP-evoked changes in nasal mucociliary transport in humans [14]. Their in vivo results, as well as our findings obtained in vitro, are in contrast to those of Lindberg et al. and Wong et al. [17,31]; both of those studies revealed considerable SP-induced stimulation of ciliary activity in vivo in animal material. Petersson et al. could stimulate nasal secretion in animals after topical SP administration, whereas in man no such effect was found [22]. This effect on secretion could possibly lead indirectly to an acceleration of mucociliary transport. This mechanism might explain the differences in human and animal mucociliary transport in vivo after administering SP.

Substance P and thiorphan

Kondo et al. demonstrated a rise in CBF by SP and thiorphan in animal material (Table 3) [16]. Their results were not confirmed in our experiments with human ciliated tissue. We found no dose-dependent change of CBF by SP and thiorphan. Several reasons may be offered for these contradictory observations. First, here too species differences may play a role. Moreover, the standard error in our results for SP together with thiorphan is notably higher than for SP alone, which contributes to our statistical outcome. In both groups, the experiments were carried out on different adenoid specimens. The measurements were performed after 5 and 10 minutes; as pointed out before, this entails a risk of overlooking earlier changes in CBF. Finally, it should be emphasized that observations in an in vitro experiment have some drawbacks and limitations. Obviously, the physiological role of SP in an in vivo situation is embedded in a complex system.

Conclusions

SP alone produces only a minor decrease in the CBF of human upper respiratory cilia, whereas SP with thiorphan showed no effect on CBF. Therefore, human mucociliary transport will probably not be influenced by a direct action of SP on ciliary activity.

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CHAPTER 7

CALCITONIN GENE-RELATED PEPTIDE STIMULATES CILIARY BEAT IN HUMAN UPPER RESPIRATORY CILIA

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SUMMARY

Calcitonin gene-related peptide (CGRP) is one of the neuropeptides that is released in the human nasal mucosa after trigeminal nerve stimulation. We have investigated the effect of CGRP on ciliary beat frequency (CBF) of human upper respiratory cilia in vitro. Ciliated epithelium of adenoids was used in the experiments. CBF was measured by a computerized photoelectrical method. CGRP showed a significant dose-dependent stimulation of CBF in concentrations of 10^{-9}-10^{-6} M (n=10), with a maximum of 23 %. We conclude that CGRP may play a role in a protective reflex of the airway epithelium in vivo by directly stimulating ciliary beat.

KEY WORDS

Calcitonin gene-related peptide - ciliary beat frequency - human upper respiratory cilia

INTRODUCTION

Mucociliary transport plays an essential role as a first line of defense in the airways. In human nasal mucosa, the presence of several neuropeptides has been demonstrated [1-5,15]. Among them are substance P, neurokinin A, and calcitonin gene-related peptide (CGRP), all of which are found in trigeminal nerve endings. After trigeminal stimulation, these neuropeptides are released as a result of a local axon reflex. The question now arises whether and to what extent these neuropeptides could influence mucosal physiology, particularly ciliary activity.

In patients with an allergic rhinitis, an increased level of CGRP was found in nasal washings after allergen challenge [10,17]. This indicates that CGRP is released by mediators of type I allergy, and may contribute to nasal symptoms. After depletion of trigeminal nerve endings from neuropeptides by local application of capsaicin, a diminished symptom score was found in non-allergic rhinitis patients [8]. Furthermore, a decrease in CGRP in the human nasal mucosa has been reported under the same conditions [8]. Patients suffering from chronic sinusitis revealed a higher level of CGRP in nasal biopsies and secretions as compared with a control group [11]. There is additional evidence that CGRP plays a role in the pathogenesis of nasal symptoms. Binding sites for this neuropeptide have been demonstrated in the walls of small muscular arteries and arterioles in the human nasal mucosa [3].

Therefore, CGRP might lead to arterial dilatation. Indeed, intranasal administration of CGRP was found to increase nasal resistance [12]. However, Wolf could not corroborate this effect [18]. Moreover, stimulation of glandular secretion by CGRP was shown in the guinea pig and human nasal mucosa [6,12].

Up till now, few studies have been published on the effect of CGRP on mucociliary function [9,14,19]. Other effects of CGRP on human nasal physiology have not been completely elucidated yet. The aim of this study was to determine the effect of CGRP on the ciliary beat frequency (CBF) of the human upper respiratory tract. Human adenoid cilia were elected because species differences in ciliary reaction have been shown [16]. An in vitro system was preferred in order to exclude possible effects on ciliary beat caused by change in the amount or composition of mucus.

MATERIALS AND METHODS

Tissue preparation

The ciliated epithelium of human adenoids was used for the experiments. The material was processed as previously described [13]. The adenoids were rinsed in 0.9 % saline solution and then transferred to the medium CMRL-1066 (Gibco, Paisley, UK) with several additives [7,20]. Pieces of approximately 0.3 cm in diameter were cut from the ciliated epithelium. The specimens were then studied microscopically in order to find a proper row of beating cilia. When such a row was found, the specimen was used for measurement.

CBF measurements

For the CBF measurements, we used the computerized photoelectrical method described by Ingels et al. [7]. The ciliated specimens were brought into a perfusion chamber that was placed on a phase-contrast microscope (Leitz, Wetzlar, Germany). Ciliary beat was measured by a photometer and digitalized by an A/D converter. A fast Fourier transform analysis of the recorded signal revealed a power spectrum. CBF was determined from the first harmonic of this power spectrum. All CBF measurements in one experiment were performed on a single ciliated cell (about 200 cilia). The temperature in the perfusion chamber was kept at 34 °C by means of an electronic heating device.

Experimental design
Initial CBF was determined after perfusion with neutral medium. CGRP (Bachem Feinchemikalien AG, Bubendorf, Switzerland) was added to the neutral medium. This caused no change in pH (7.4) because of the buffer capacity of the medium. Next, increasing concentrations of CGRP were perfused in the chamber (10^{-9}-10^{-6} M, n=10). CBF recordings were made 5 and 10 min after discontinuation of perfusion of each concentration. After the measurements with each concentration of CGRP, a wash-out procedure with neutral medium was performed. CBF was recorded once again after 5 min after this wash-out procedure.

Statistical analysis
Statistical analysis was carried out by analysis of variance (ANOVA). A p value < 0.05 was considered significant. All values are expressed as the mean ± SEM.

RESULTS

The results are shown in Table 1 and Figures 1, 2 and 3. Administration of CGRP resulted in a significant dose-dependent stimulation of CBF, both for the measurements after 5 min (ANOVA, $p = 0.028$) and after 10 min (ANOVA, $p = 0.029$). At the maximum concentration (10^{-6} M), the increase in CBF amounted to 17.6 % after 5 min and 23.4 % after 10 min. After washing-out with neutral medium, no significant effect could be found anymore.

Table 1 Effect of CGRP on CBF (mean ± SEM), n=10. CBF measurements made after discontinuation of perfusion with CGRP (5 and 10 min) and with neutral medium (5 min).

Concentration (M)	CBF ± SEM (Hz) t=5 min	CBF ± SEM (Hz) t=10 min	CBF ± SEM (Hz) after neutral medium
Initial	7.8 ± 0.5		
10^{-9}	7.9 ± 0.4	8.4 ± 0.7	8.6 ± 0.6
10^{-8}	9.2 ± 0.7	8.3 ± 0.5	8.2 ± 0.7
10^{-7}	9.2 ± 0.5	8.2 ± 0.8	8.2 ± 0.6
10^{-6}	9.1 ± 0.6	9.6 ± 0.7	8.3 ± 0.5

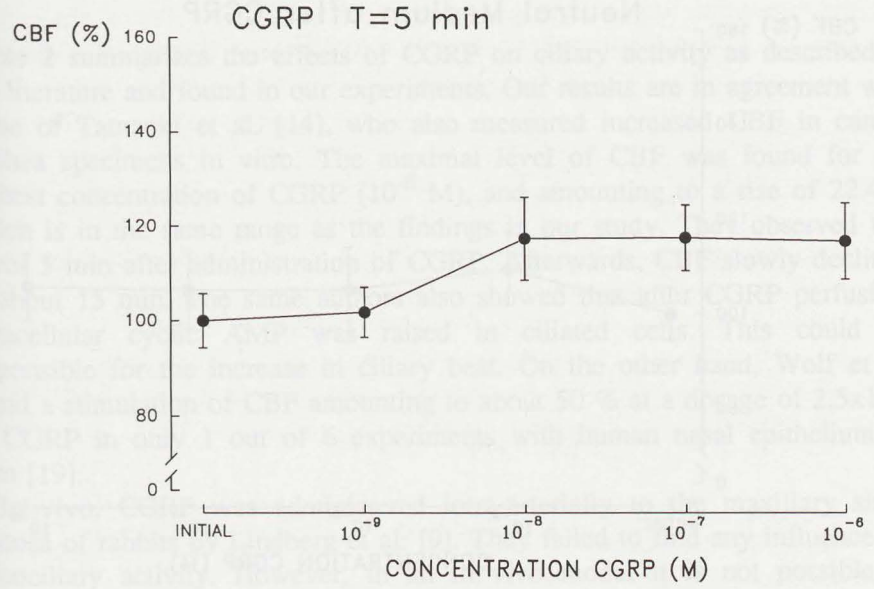

Figure 1 Effect of CGRP on mean CBF ± SEM (n=10), 5 min after discontinuation of perfusion. Initial CBF (7.8 Hz) is set at 100%.

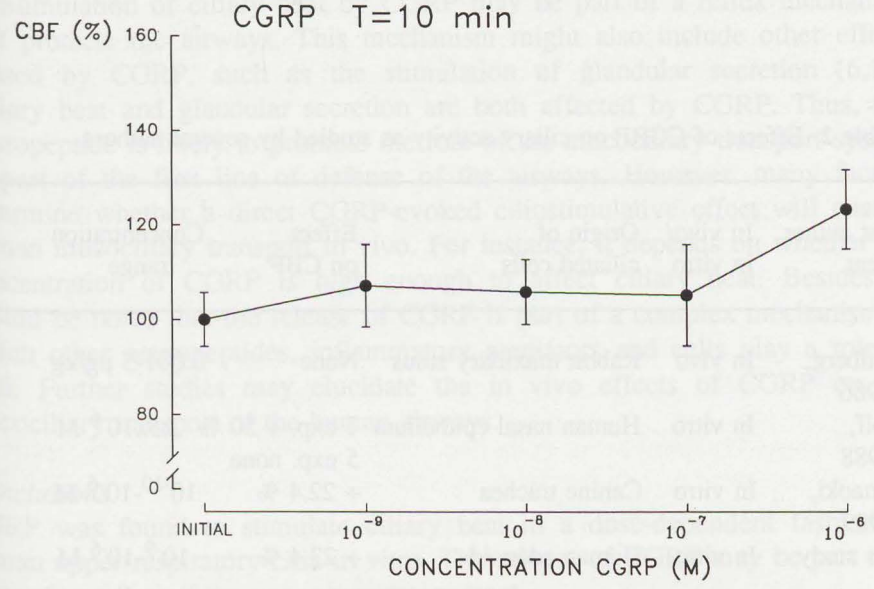

Figure 2 Effect of CGRP on mean CBF ± SEM (n=10), 10 min after discontinuation of perfusion. Initial CBF (7.8 Hz) is set at 100%.

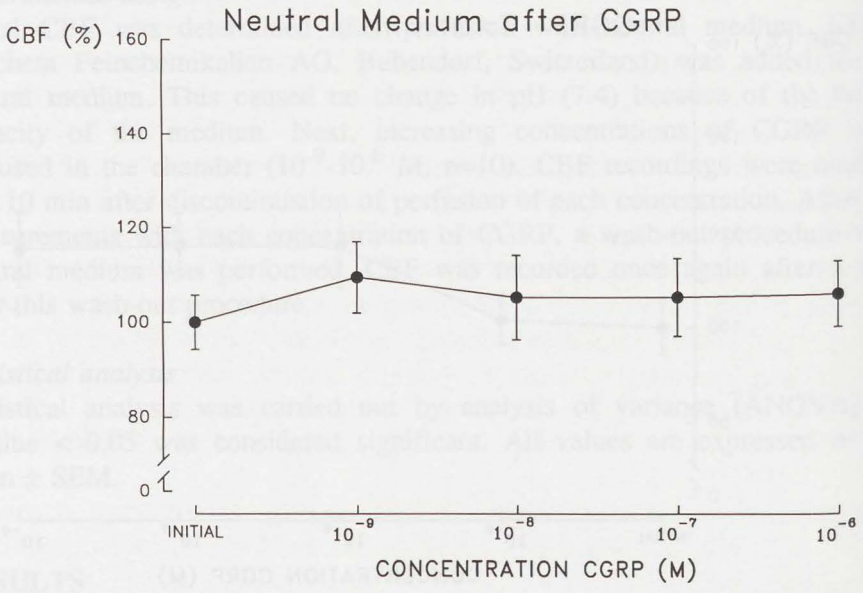

Figure 3 Effect of neutral medium after CGRP perfusion on mean CBF ± SEM (n=10), 5 min after discontinuation of perfusion with neutral medium. Initial CBF (7.8 Hz) is set at 100%.

Table 2 Effects of CGRP on ciliary activity as studied by several authors.

First author, year	In vivo/ in vitro	Origin of ciliated cells	Effect on CBF	Concentration range	n
Lindberg, 1986	In vivo	Rabbit maxillary sinus	None	0.001-5 µg/kg	5
Wolf, 1988	In vitro	Human nasal epithelium	1 exp. + 50 % 5 exp. none	2.5×10^{-6} M	6
Tamaoki, 1989	In vitro	Canine trachea	+ 22.4 %	10^{-10}-10^{-6} M	7
This study	In vitro	Human adenoid	+ 23.4 %	10^{-9}-10^{-6} M	10

n = number of experiments

DISCUSSION

Table 2 summarizes the effects of CGRP on ciliary activity as described in the literature and found in our experiments. Our results are in agreement with those of Tamaoki et al. [14], who also measured increased CBF in canine trachea specimens in vitro. The maximal level of CBF was found for the highest concentration of CGRP (10^{-6} M), and amounting to a rise of 22.4%, which is in the same range as the findings in our study. They observed this effect 5 min after administration of CGRP. Afterwards, CBF slowly declined in about 15 min. The same authors also showed that after CGRP perfusion, intracellular cyclic AMP was raised in ciliated cells. This could be responsible for the increase in ciliary beat. On the other hand, Wolf et al. found a stimulation of CBF amounting to about 50 % at a dosage of 2.5×10^{-6} M CGRP in only 1 out of 6 experiments with human nasal epithelium in vitro [19].

In vivo, CGRP was administered intra-arterially to the maxillary sinus mucosa of rabbits by Lindberg et al. [9]. They failed to find any influence on mucociliary activity. However, in an in vivo model it is not possible to distinguish between a direct or indirect effect on ciliary beat. Furthermore, species differences could also play a role. These might explain the different findings reported in the studies published so far.

Stimulation of ciliary beat by CGRP may be part of a reflex mechanism that protects the airways. This mechanism might also include other effects caused by CGRP, such as the stimulation of glandular secretion [6,12]. Ciliary beat and glandular secretion are both affected by CGRP. Thus, this neuropeptide is likely to promote the role of the mucociliary transport system as part of the first line of defense of the airways. However, many factors determine whether a direct CGRP-evoked ciliostimulative effect will change human mucociliary transport in vivo. For instance, it depends on whether the concentration of CGRP is high enough to affect ciliary beat. Besides, it should be noted that the release of CGRP is part of a complex mechanism in which other neuropeptides, inflammatory mediators and cells play a role as well. Further studies may elucidate the in vivo effects of CGRP on the mucociliary transport of the human airways.

Conclusions

CGRP was found to stimulate ciliary beat in a dose-dependent fashion in human upper respiratory cilia in vitro. The release of CGRP may be part of a protective reflex of the upper respiratory tract.

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CHAPTER 8

SUMMARY AND CONCLUSIONS

INTRODUCTION

Mucociliary transport in the respiratory tract is an essential part of the body's protection against intrusion by noxious substances. Ciliary beat is the major driving force of this mechanism. Another important factor is the mucus covering the epithelium of the airways. If these factors of transport become impaired, mucociliary clearance will be diminished or absent. As a consequence, infections may occur, while the symptoms of type I allergy and hyperresponsiveness syndromes may be enhanced. Some well-known disorders in which mucus production is disturbed or ciliary activity affected are cystic fibrosis and Kartagener's syndrome, respectively. The latter condition is now considered a form of primary ciliary dyskinesia.

Our present knowledge about mucociliary transport and its 2 major factors is still far from complete. There are considerable gaps in our understanding of normal ciliary physiology and the production of the mucus layer. Moreover, we do not yet know exactly how the human respiratory mucosa is influenced by various compounds released in infection, allergy, and hyperresponsiveness of the airways.

Several mediators are known to play a key role in the pathogenesis of allergic rhinitis. After nasal allergen challenge, nasal secretions have a higher concentration of certain mediators of allergy. Furthermore, patients with allergic rhinitis may exhibit a lower level of mucociliary transport and ciliary beat.

A number of neuropeptides are present in the airways. Stimulation of sensory trigeminal nerves triggers the release of neuropeptides in the upper respiratory mucosa, including substance P (SP), calcitonin gene-related peptide (CGRP), and neurokinin A. These neuropeptides are liberated from these nerve endings as a local axon reflex. Moreover, mediators of allergy can also trigger the release of these neuropeptides. The latter contribute to the symptoms of patients with hyperresponsiveness to nonspecific stimuli, as in the case of non-allergic rhinitis. Little is known about their influence on ciliary activity of the human upper airways.

In the present study, we have gathered information about how certain mediators of allergy and neuropeptides exert a direct effect on the ciliary activity of the human upper respiratory tract. Changes in ciliary activity may alter mucociliary transport. This, in turn, can contribute to the symptoms in allergic and non-allergic rhinitis.

LITERATURE

Chapter 2 gives an overview of the present state of knowledge about cilia. Attention was focused on the upper respiratory tract. The morphology of ciliated mucosa and its presence in the human body were described, as well as the ciliary ultrastructure and physiology. Furthermore, we discussed certain influences on ciliary beat, some of the properties of the mucus, and the ways in which ciliary beat and mucociliary transport can be assessed. Several disorders found in the upper respiratory tract and related mucociliary pathology were dealt with. Finally, the known effects of mediators of allergy and neuropeptides on mucociliary function were considered.

METHODS

The main parameter expressing ciliary activity is ciliary beat frequency (CBF). CBF can be measured in 2 ways: by reflected light or by transillumination. In the first technique, light reflected from cilia can be converted into a sinusoidal signal, from which their beating frequency can be determined. This technique can be applied either in vitro or vivo, the latter mainly in animals. In vivo investigation has a disadvantage: effects caused by change in the amount or composition of mucus may influence the CBF measurements. Transillumination is an in vitro technique and is the one most used nowadays. This is the technique we chose for our experiments.

The procedure involved registration of any variation in light intensity produced by the beating cilia of an epithelial specimen that is placed in the light beam of a phase-contrast microscope. These variations were detected by a photoelectrical cell, then digitalized, and input into a personal computer. A fast Fourier transform analysis of the recorded sinusoidal signal was performed by the computer. This analysis revealed a power spectrum from which the CBF could be determined. All CBF measurements in an experiment were performed on a single ciliated cell (containing about 200 cilia), since CBF can vary between different cells in the same specimen. We only examined ciliated cells that were adjacent to others, and exhibited cilia beating freely. Furthermore, a second possible parameter of ciliary activity could be derived from the sinusoidal signal. The consistency of this signal was expected to be an indication of the ciliary beating harmony, which may be related to ciliary co-ordination. This parameter is called signal consistency (SC).

In our experiments, we put specimens of ciliated upper respiratory epithelium into a perfusion chamber that was placed on the stage of a phase-

contrast microscope. In this chamber, the temperature was kept at 34 °C. It was important to maintain a constant level during the experiments, as CBF is temperature-dependent. The perfusion chamber was filled with the medium CMRL-1066, which contained several additives and had a physiological pH. It is known that CBF can be influenced by a pH that lies outside the range of physiological values.

The experimental set-up was as follows. The initial CBF was determined after perfusion only with medium. Subsequently, the lowest concentration of the substance studied was perfused and CBF was measured after 5 and 10 min. Then, a wash-out perfusion with medium was performed, and CBF was determined again. The procedure was repeated for increasing concentrations of the same compound. In this way, a number of experiments with the same substance, but performed on different ciliated specimens, could be conducted. Statistical analysis was carried out by analysis of variance (ANOVA) in order to determine if a significant dose-dependent effect was present.

MATERIALS

To study the effect of several substances on ciliary activity, the use of human material is preferred. It has been shown that different effects may occur in various species. In this study, we concentrated on the effects of various compounds that are known to be released in patients with nasal disorders. Therefore, the use of human ciliated tissues was indispensable. For in vitro function studies, we had to have human upper respiratory ciliated tissue at our disposal, whereby the ciliary activity was most comparable to the normal physiological CBF. Furthermore, ciliated tissue had to be amply available. This issue was discussed in *Chapter 3*. We investigated 2 potential sources of human upper airway ciliated material: one is preserved respiratory epithelium from nasal polyp mucosa and inferior turbinate mucosa brought into cell suspension culture; and the other is epithelium from freshly obtained adenoids. CBF and SC were determined. The time scale for measurements differed for both sources.

The formation of aggregates took 1 week in the preservation technique. At that moment CBF could be measured, but a non-physiological value (5.8 Hz) was found. Hereafter, CBF was determined at weekly intervals and could even be assessed after 7 weeks. However, it remained at this low level. Also SC decreased in time, although less dramatically than CBF. Therefore, we regard cilia in cell suspension culture as less appropriate for function experiments. Accordingly, this material was not used in our investigations.

The ciliated epithelium of the adenoid tissue showed cilia beating at an almost constant frequency as initially (9.3 Hz) for a period of 5 hours. SC, however, decreased spontaneously with time. As CBF proved to be stable, we chose ciliated epithelium of adenoids for our experiments. It was all the more preferable since this tissue is readily available in ENT practice. SC was not determined in further experiments; the value of this parameter is still unclear.

EFFECTS OF MEDIATORS OF ALLERGY ON CILIARY BEAT FREQUENCY

Histamine, leukotriene C_4

Chapter 4 was devoted to the influence of histamine and leukotriene C_4 (LTC_4) on CBF. Our investigation measured the effect of increasing concentrations of these mediators. Neither histamine nor LTC_4 showed a significant dose-dependent effect.

Histamine, however, is known to increase mucociliary transport in vivo. Obviously, this is not caused by a direct effect on ciliary activity. Instead, the increased transport might be a result of greater vasopermeability or a change in mucus secretion. Alternatively, it might be due to the release of other mediators, such as prostaglandins, and of the neuropeptide SP under the influence of histamine.

As far as LTC_4 is concerned, the literature contains contradictory findings. Both stimulation and depression of CBF have been reported. However, we should bear in mind that some of the results are not comparable because of methodological differences among the diverse studies. In our investigation, we did not find any direct dose-dependent effect of LTC_4 on CBF.

Prostaglandins D_2 and E_2

Chapter 5 dealt with the way increasing doses of prostaglandin D_2 (PGD_2) and E_2 (PGE_2) affect CBF. In our study, PGD_2 appeared to have no direct effect on CBF. This is a remarkable result, because PGD_2 is released in the early phase of a type I allergic reaction and is considered an important mediator in allergic rhinitis. PGE_2, on the contrary, did induce a significant dose-dependent increase in CBF. This finding is in accordance with the sparse data in the literature. PGE_2 probably raises the level of intracellular cyclic AMP, a known determinant of CBF.

The 4 mediators of allergy studied here had no direct depressing effect on ciliary activity. Thus, it seems that in the case of allergic rhinitis, a decrease in mucociliary transport is not a direct effect on ciliary activity of increased levels of these mediators. Other mechanisms may play a role. These include altered mucus properties, the action of other mediators, the inflammatory reaction resulting from the allergic cascade, or a combination of these events. The finding that PGE_2 is able to increase ciliary activity could be important in improving mucociliary transport in vivo.

EFFECTS OF NEUROPEPTIDES ON CILIARY BEAT FREQUENCY

Substance P

Chapter 6 investigated the influence of SP on ciliary beat. We were able to demonstrate a minor, though significant decrease in CBF. However, we did not find this effect when the specimen was perfused with SP in combination with thiorphan, which is a blocker of the SP-degrading enzyme neutral endopeptidase.

SP is able to produce nasal obstruction and it is thought to heighten secretory activity. Furthermore, the literature reports a stimulating effect of SP on the in vivo mucociliary transport in animals. However, the nasal mucociliary transport in humans was not changed by SP. Moreover, our data gives no decisive ground to suggest that a possible change in mucociliary clearance is caused by a direct effect of SP on ciliary activity.

Calcitonin gene-related peptide

Chapter 7 reported our findings regarding the effect of CGRP on CBF. We found a significant dose-dependent increase, whereby CBF rose by a maximum of 23%.

The scarce data in the literature point to a stimulative role of CGRP on glandular secretion. Little information exists on how CGRP affects human ciliary activity. The substantial rise in CBF that we found is apparently a direct effect of CGRP on the beating cilia. This effect, in combination with the stimulative influence of CGRP on secretion, suggests that CGRP might promote mucociliary transport as part of the defensive mechanisms of the airways. However, the release and influence of CGRP is embedded in a complex system in which many elements play a role.

FINAL REMARKS

In this study, we determined the effects of a number of substances on human upper respiratory ciliary activity in vitro. In future, attention should primarily be focused on substances that are assumed to be involved in allergy and hyperresponsiveness.

Furthermore, techniques have recently become available whereby the ciliary activity can be determined in humans in vivo. It would be enlightening if pharmacological studies were to apply these techniques too. Then, the results from both in vitro and in vivo studies could be combined. On that basis, we might be able to distinguish whether the effects on ciliary activity are direct or indirect. Indirect effects on ciliary function could be brought about by altered composition or amount of mucus. The ultimate goal of future studies would be to further determine the extent to which the mucociliary transport system contributes to the defensive mechanisms in the human respiratory tract, both in physiological and pathological conditions.

SAMENVATTING

SAMENVATTING

Het mucociliaire transport in de luchtwegen is een essentieel deel van de afweer van het lichaam tegen mogelijk schadelijke invloeden. De trilhaarslag is de voortstuwende kracht van dit transport. Ook het slijm dat het epitheel van de luchtwegen bedekt is een belangrijk element. Bij afwijkingen in trilhaarfunctie of slijmproduktie kan het mucociliaire transport verstoord raken. Dit kan vervolgens aanleiding geven tot luchtweginfecties. Bovendien kunnen symptomen van bijvoorbeeld allergie of hyperreactiviteit van de luchtwegen versterkt worden. Afwijkingen, waarbij de slijmproduktie of de trilhaaractiviteit gestoord is, zijn bijvoorbeeld mucoviscoidosis (cystic fibrosis) en het syndroom van Kartagener. Dit laatste ziektebeeld wordt tegenwoordig beschouwd als een vorm van primaire ciliaire dyskinesie.

De kennis over het mucociliaire transport en zijn twee belangrijkste determinanten is nog verre van compleet. Ook is nauwelijks bekend hoe dit transport beïnvloed kan worden door stoffen die vrijgemaakt worden tijdens infectie, allergische reactie of hyperreactiviteit. Van patiënten met een allergie van de luchtwegen is bekend dat het mucociliaire transport verminderd kan zijn. In de allergische reactie wordt een groot aantal mediatoren in het slijmvlies vrijgemaakt. Deze zouden mogelijkerwijs voor deze vermindering verantwoordelijk kunnen zijn. Bij patiënten met hyperreactiviteit is de concentratie van een aantal neuropeptiden in het luchtwegepitheel verhoogd. Er is weinig bekend over de invloed van deze stoffen op het mucociliaire transport.

In het onderzoek beschreven in dit proefschrift is nagegaan of een aantal mediatoren en neuropeptiden een direct effect kan uitoefenen op de activiteit van trilharen van de bovenste luchtwegen. Verandering in deze activiteit kan gevolgen hebben voor het mucociliaire transport, waardoor symptomen van allergie of hyperreactiviteit duidelijker op de voorgrond kunnen treden. De opzet van het onderzoek was als volgt. Met behulp van een foto-elektrische registratiemethode werd in vitro de trilhaarslagfrequentie (ciliary beat frequency, CBF) gemeten van trilhaardragende cellen van de bovenste luchtwegen. Tevens werden veranderingen van deze CBF onder invloed van mediatoren en neuropeptiden bepaald. Wij zijn er hierbij vanuit gegaan - evenals andere onderzoekers - dat de CBF beschouwd kan worden als een belangrijke parameter van de ciliaire activiteit.

In *Hoofdstuk 2* wordt een overzicht gegeven van de huidige kennis omtrent trilharen, in het bijzonder die van de bovenste luchtwegen. De morfologie van het trilhaardragend epitheel wordt beschreven, alsook de ciliaire ultrastructuur en fysiologie. Daarnaast wordt besproken hoe de trilhaarslag beïnvloed kan

worden. Enige eigenschappen van het slijm worden vermeld, evenals de methoden waarop de trilhaarslag en het mucociliaire transport gemeten kunnen worden. Verschillende afwijkingen van de bovenste luchtwegen en de hieraan gerelateerde mucociliaire afwijkingen worden belicht. Ten slotte wordt een beschouwing gegeven van al bekende effecten van mediatoren en neuropeptiden op de mucociliaire functie.

In *Hoofdstuk 3* wordt een studie beschreven van een tweetal in vitro onderzoeksmodellen van trilhaardragend epitheel van de menselijke bovenste luchtwegen. Het is bekend dat de reactie van trilharen verschillend kan zijn tussen mensen en diverse diersoorten. Het onderzoek van dit proefschrift heeft betrekking op de invloed van stoffen die vrijkomen bij aandoeningen van de menselijke luchtwegen. Derhalve verdient het gebruik van menselijk materiaal hierbij de voorkeur.

In het eerste model werden trilhaarcellen in suspensie gebracht, die vervolgens aggregaten van cellen vormden. Het voordeel van dit model is dat deze aggregaten enige weken tot maanden met een functionerende trilhaarslag in medium kunnen overleven. De tijdsduur voor de vorming van de aggregaten bedroeg 1 week, zodat hierna de CBF gemeten kon worden. Echter, op dat moment bleek de CBF al op een niet-fysiologisch niveau te zijn (5,8 Hz). De CBF werd vervolgens met intervallen van 1 week bepaald en bleek na 7 weken nog meetbaar. De CBF bleef echter op ongeveer hetzelfde lage peil als na 1 week. Een van de CBF-meting afgeleide parameter is de signaal consistentie (SC), die mogelijk een aanduiding is van ciliaire coördinatie. Ook deze bleek in de loop van de tijd af te nemen, alhoewel niet in dezelfde mate als de CBF. Vanwege de niet-fysiologische CBF in dit model zijn wij van mening dat het minder geschikt is voor het verrichten van onderzoek naar de trilhaarfunctie.

In het tweede model werd gebruik gemaakt van trilhaardragend epitheel van adenoid, kort na adenotomie. De CBF bleek gedurende 5 uur op hetzelfde niveau te blijven als de uitgangswaarde (9,3 Hz). De SC daalde in deze periode.

Op grond van deze uitkomsten werd gekozen voor gebruik van adenoidepitheel in de experimenten vanwege de constante fysiologische CBF en de ruime beschikbaarheid. In de studie werd de SC verder niet bepaald, aangezien de betekenis ervan vooralsnog niet geheel duidelijk is.

In *Hoofdstuk 4* wordt beschreven welke invloed de mediatoren histamine en leukotriene C_4 (LTC_4) op de CBF hebben. In dit onderzoek werden effecten bepaald van toenemende concentraties van deze mediatoren. Noch histamine, noch LTC_4 vertoonde een significant dosis-afhankelijk effect.

In *Hoofdstuk 5* worden de resultaten weergegeven van de studie naar de effecten van de mediatoren prostaglandine D_2 (PGD_2) en prostaglandine E_2 (PGE_2) op de CBF. PGD_2 vertoonde geen significante invloed. Voor PGE_2 daarentegen werd een dosis-afhankelijke toename van de CBF gevonden, met een maximum van 37%.

Geen van het viertal door ons onderzochte mediatoren toonde een direct negatief effect op de CBF in vitro. Een verminderd mucociliair transport, zoals dat kan voorkomen bij allergische rhinitis, lijkt dan ook niet teweeg gebracht te worden door een directe invloed van deze mediatoren op de trilhaaractiviteit. Andere mechanismen spelen hierbij mogelijk wel een rol, zoals een verandering van de hoeveelheid of samenstelling van het slijm, een effect van andere mediatoren of neuropeptiden, een ontstekingsreactie of combinaties hiervan. De gevonden stimulerende invloed van PGE_2 op de CBF kan van belang zijn voor een verbeterd mucociliair transport in vivo.

In *Hoofdstuk 6* wordt de invloed van het neuropeptide substance P (SP) op de CBF beschreven. SP gaf een kleine - maar statistisch significante - afname van de CBF te zien. Ook werd het effect van SP in combinatie met thiorphan onderzocht. Thiorphan is een stof die een enzym dat SP afbreekt (neutral endopeptidase) blokkeert. In dit experiment kon geen effect op de CBF worden aangetoond. Het lijkt dan ook niet waarschijnlijk dat SP het mucociliaire transport in de menselijke luchtwegen verandert door een direct effect op de trilhaarslag.

In *Hoofdstuk 7* wordt het effect van het neuropeptide calcitonine gene-related peptide (CGRP) op de CBF besproken. CGRP bleek een significante dosis-afhankelijke stimulatie te bewerkstelligen, met een maximum van 23%. Aangezien CGRP ook slijmsecretie stimuleert, zou het beschouwd kunnen worden als een promotor van het mucociliaire transport.

In *Hoofdstuk 8* worden de resultaten van de verschillende studies samengevat en nader besproken.

SLOTOPMERKINGEN

In het onderzoek beschreven in dit proefschrift werd gebruik gemaakt van een in vitro techniek om de effecten van verschillende mediatoren en neuropeptiden op de activiteit van trilharen van de bovenste luchtwegen vast te stellen. Nader onderzoek zou met name gericht dienen te zijn op andere substanties, waarvan verondersteld kan worden dat ze een rol spelen bij allergie of hyperreactiviteit.

Daarnaast komen er op dit moment methoden beschikbaar om trilhaaractiviteit in vivo te bepalen bij menselijke proefpersonen. Zodra op deze wijze ook farmacologische invloeden goed bestudeerd kunnen worden is het mogelijk de resultaten van in vitro en in vivo onderzoek te combineren. Dan kan beter bepaald worden of effecten, uitgeoefend op de ciliaire activiteit, van directe of indirecte aard zijn. Indirecte effecten zouden teweeg gebracht kunnen worden door een verandering van de samenstelling of van de hoeveelheid slijm. Het uiteindelijke doel van toekomstig onderzoek zou gericht moeten zijn op een nadere precisering van de mate waarin het mucociliaire transport bijdraagt aan het complexe geheel van afweermechanismen van de luchtwegen, zowel onder fysiologische als pathologische omstandigheden.

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