Effects of Pharmaceutical Compounds on Ciliary Beating in Human Nasal Epithelial Cells: A Comparative Study of Cell Culture Models

Remigius Uchenna Agu,[1] Mark Jorissen,[2] Tom Willems,[2] Guy Van den Mooter,[1] Renaat Kinget,[1] and Patrick Augustijns[1,3]

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Purpose. To test two *in vitro* human nasal epithelial cell culture systems for their ability to screen the effects of pharmaceutical compounds on ciliary beating.

Methods. Human nasal epithelial cells were cultured as monolayer and in a sequential monolayer-suspension culture with *in vitro* ciliogenesis. The influence of reference cilio-stimulatory compounds (glycocholate, isoprenaline), reference cilio-inhibitory compounds (chlorocresol, diphenhydramine) and pH on ciliary beating was investigated using computerized microscope photometry.

Results. Sodium glycocholate (0.5% w/v) maximally and reversibly increased CBF of the cells in both culture systems by 26 ± 4% (monolayer) and 18 ± 6% (suspension). Similarly, isoprenaline (10^{-3} M) maximally, but irreversibly increased CBF of the cells by 14 ± 3% (monolayer) and 17 ± 4% (suspension). Chlorocresol (0.005% w/v) reversibly reduced the CBF of the cells by 50 ± 6% (monolayer) and 34 ± 4% (suspension); at a higher concentration (0.1% w/v) it resulted in instantaneous and irreversible ciliostasis. Diphenhydramine (0.1% w/v) reversibly reduced CBF in both culture systems by 45 ± 13% (monolayer) and 69 ± 5% (suspension); irreversible cilio-stasis occurred in less than 2 minutes in both culture systems upon cell exposure to diphenhydramine (1.0% w/v). In the monolayer culture system, CBF was stable only within the physiological pH range of 6.5–8.0; ciliary beating in the suspension culture remained stable within a pH range of 4.0–10.0.

Conclusions. Both cell culture systems are suitable for screening the effects of pharmaceutical compounds on ciliary beating. Especially the sequential monolayer-suspension culture appears to be very promising as ciliary activity can be preserved for as long as 6 months.

KEY WORDS: human nasal epithelium; ciliary beat frequency; cilio-stimulation; cilio-inhibition; pharmaceutical compounds.

INTRODUCTION

Although the oral route is the most attractive mode of drug administration, it does not always result in acceptable bioavailability due to pre-systemic metabolism or limited transport across the intestinal membranes, necessitating the development of parenteral formulations. Recently intranasal administration of drugs has been explored as an alternative to parenteral injections. Especially for the administration of proteins and peptides, this administration route has been shown to be very efficient (e.g., oxytocin, vasopressin).

It is obvious that, when nasal administration of drugs is considered for systemic and/or local action, the effect of the drug and additives on ciliary movement (the motor of mucociliary transport) is investigated in an early stage of drug development (1,2). Several methods employing fresh ciliated tissue from different species have been described to measure ciliary beat frequency (CBF) *in vitro*. The use of fresh tissues has, however, several limitations. It is necessary to process the specimens soon after collection as ciliary activity may deteriorate rapidly (3). In addition, investigations using fresh samples are limited in time because of the risk of bacterial contamination and the inability to keep the cells in good condition for days (4). A direct effect of anesthesia and surgical trauma on CBF of human nasal tissues can also not be excluded. *In vitro* human nasal epithelial cell models in which above problems are addressed are contemporary research issues, especially with respect to preservation of ciliary activity for a long period of time.

The objectives of this study were related to the evaluation and comparison of two human *in vitro* models to screen the effect of various compounds on ciliary beat frequency. In the first system, human nasal epithelial cells from excised mucosal tissue were cultured as monolayer with preservation of ciliary activity during up to one week; in the second system, a sequential monolayer-suspension culture technique that enables ciliogenesis *in vitro* was used. This system allows preservation of CBF for months.

MATERIALS AND METHODS

Chemicals

Sodium glycocholate and isoprenaline.HCl were supplied by Sigma (St. Louis, MO, USA). Chlorocresol and diphenhydramine.HCl were obtained from UCB (Leuven, Belgium) and Triamedico (Sint-Niklaas, Belgium), respectively.

Cell Culture Procedures

Cells were cultured as described previously (1,4). Briefly, after enzymatic dissociation of human nasal epithelial cells using 0.1% pronase (Sigma, St. Louis, MO, USA), cells were pre-plated on plastic to reduce fibroblast contamination. For the monolayer culture, the cells were seeded at a density of 5×10^5 cells/cm^2 in a 24-well cell culture plate containing cover glasses (∅13mm). The monolayer culture medium [2% Ultroser G in DMEM-F12 (Life Laboratories, Paisley, UK)] was changed the day after seeding and subsequently 3 times a week.

For the sequential monolayer-suspension culture, cells were plated at a density of 5×10^5 cells/cm^2 in T 75 tissue culture flasks (Falcon, Oxnard, CA, USA) coated with 0.2% collagen gel (extracted from rat tails). The monolayer culture medium was changed the following day and subsequently three times a week. After 3 weeks in the monolayer culture, the cells were released from the collagen gel with 200 IU/ml-collagenase type IV (Worthington Biochemical Corporation, Freehold, NJ, USA). The resulting cells were subsequently cultured in several

[1] Laboratorium voor Farmacotechnologie en Biofarmacie, K.U. Leuven, Campus Gasthuisberg O&N, Herestraat 49, B-3000 Leuven, Belgium.

[2] Laboratorium voor Experimentele Otorhinolaryngologie, K.U. Leuven, Campus Gasthuisberg O&N, Herestraat 49, B-3000 Leuven, Belgium.

[3] To whom correspondence should be addressed. (e-mail: patrick.augustijns@med.kuleuven.ac.be)

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T 25-tissue flasks. The following weeks, the medium consisted of 10.0% NU-serum in DMEM-F12 1/1 (Life Laboratories, Paisley, UK). Cells in both culture systems were incubated at 37°C in a 5% CO_2 atmosphere.

Preparation of Solutions

Unless stated otherwise, solutions were prepared by dissolving the required amount of the compound in Ham's DMEM-F12 1/1, pH 7.3 or by spiking with a stock solution of the substance. The final concentrations were expressed in molar (M) or percentage weight-volume (% w/v). Isoprenaline was prepared in a hermetically sealed vial bubbled with nitrogen to reduce atmospheric oxidation. DMEM-F12 1/1 served as a control solution for all CBF measurements.

For experiments in which the effect of pH on CBF was studied, the pH modification of DMEM-F12 1/1 (2.0–12.0) was achieved using HCl (1.0%) or NaOH (1.0%). The use of buffers was avoided to exclude the additional effect of buffer species.

Ciliary Beat Frequency Studies

The CBF of the cultured cells was determined by computerized microscope photometry.

The influence of pH, drugs and excipients on the CBF of cells cultured as monolayer was studied using a perfusion method. The glasses covered with cells cultured as monolayer were placed in a 0.3 ml perfusion well. The well was subsequently placed on an invert microscope stage (40× objective and 12.5× ocular, Labovert, Leitz, Germany) and perfused with the control solution for 10 minutes. The microscope was placed on a vibration free table (Barry Controls). After the perfusion, CBF of 30 individual cells was measured in three batches. Upon perfusion with test solution, CBF measurement of the same cells was repeated. Measurements were started 2 minutes after perfusion was stopped to reduce the effects of mechanical stimulation on CBF.

The CBF of cells in the suspension culture was determined in T25 tissue culture flasks. In order to perform the measurements, the control and test solutions were removed just before the measurement, leaving the cell aggregates adhered to the tissue flask. The CBF of different cells was measured before and after test solution exposure. Reversibility of the effect due to compounds was determined by washing the cells with DMEM-F12 1/1 for 10 minutes after test compound exposure.

The influence of pH on CBF was assessed by determining the CBF of the same (monolayer culture) or different (suspension culture) cells before and after exposure to solutions of modified pH. The pH of control solution after cell exposure was determined at the end of each experiment.

A sampling frequency of 500Hz was used to measure a signal for a period of 1 minute. The recorded signal was analyzed by performing time spectral analysis using Fast Fourier Transform on the waveform obtained.

The periods of compound exposure to cells were based on literature data and preliminary experiments carried out in our laboratory. Depending on the compound being investigated, the exposure periods ranged from 5 to 45 minutes. The effects of reference compounds on CBF with time were classified as: rapid (effect within 10 minutes), slow (effect after 10–30 minutes) and delayed (effect after 30 minutes). The degree of CBF change was classified as: mild (<10%), moderate (10–20%, reference cilio-stimulatory/inhibitory compounds) and severe (>20%, reference cilio-inhibitory compounds) or pronounced (>20%, reference cilio-stimulatory compounds). Reversibility of effect (after washing) was classified relative to CBF before compound exposure (control) as: irreversible (<50%), partially reversible (50–90%), and reversible (>90%) for reference cilio-inhibitory compounds. For reference cilio-stimulatory compounds, CBF between 90–110% or >110% of control after washing was considered reversible or irreversible, respectively.

All measurements were conducted at room temperature.

Statistical Analysis and Data Presentation

In each of the conditions investigated, the CBF of 30 individual cells was measured (in three batches of 10 cells per batch) for the control and treated groups. A total of 10 CBF data was obtained per cell for each concentration investigated in each of the groups. The mean CBF of the treated groups was expressed as a percentage of CBF value of the control group ± SEM (n = 30 cells). Differences between control and treated populations were determined by Wilcoxon's signed-rank test (monolayer culture) and Mann-Whitney U test for unpaired data (suspension culture).

RESULTS

Effects of Reference Cilio-Stimulatory Compounds on CBF

Glycocholate caused a concentration dependent increase in CBF of cells in the monolayer and suspension culture systems. At a concentration of 0.005% w/v and after 5 minutes and 20 minutes exposure, no significant effect ($p > 0.05$) on CBF was observed in both culture systems; upon increasing the concentration of glycocholate to 0.5% w/v, a pronounced increase in CBF was found in the monolayer culture. A moderate effect was observed in the suspension culture system. After 5 minutes exposure, CBF significantly increased by 26 ± 2% and 18 ± 3% for monolayer and suspension cultures, respectively. Exposure for 20 minutes resulted in 25 ± 2% and 13 ± 4% increase of CBF for the monolayer and suspension cultures, respectively. The CBF increase in both cell culture systems was rapid and stable throughout the duration of CBF measurement. The cilio-stimulatory effect of glycocholate was reversible on washing the cells with DMEM-F12 1/1.

A concentration dependent moderate increase in CBF was observed in both culture systems upon exposure to isoprenaline. After a drug-cell contact time of 30 minutes, isoprenaline (10^{-5} M) resulted in 10 ± 2% and 13 ± 3% CBF increase in monolayer and suspension culture systems, respectively. Similarly, ciliary beating increased mildly by 9 ± 2% (monolayer) and moderately by 11 ± 3% (suspension culture) on increasing the incubation period to 45 minutes. A slight increase in ciliary beating was seen on increasing the concentration of isoprenaline to 10^{-3} M. At this concentration, CBF increased moderately by 11 ± 2% and 17 ± 8% for the monolayer and suspension culture systems, respectively after 30 minutes of incubation. The cilio-stimulatory effect of isoprenaline in both culture systems was slow in onset and irreversible.

Effects of Reference Cilio-Inhibitory Compounds on CBF

Chlorocresol resulted in rapid and concentration dependent decrease of CBF in monolayer and suspension culture systems. The cilio-toxicity of this compound was severe in both culture systems. At a concentration of 0.005% w/v, chlorocresol resulted in a higher CBF reduction in the monolayer culture system ($-46 \pm 3\%$ and $-51 \pm 3\%$) as compared to the suspension culture system ($-33 \pm 2\%$ and $-34 \pm 2\%$) after exposure to the compound for 5 and 20 minutes, respectively. The CBF decrease elicited by chlorocresol in both culture systems was rapid and stable up to 20 minutes. At a concentration of 0.005% w/v and after 20 minutes exposure, chlorocresol resulted in ciliary dyskinesia in cells of both culture systems. The cilia of the cells exposed to the compound were observed to beat in an uncoordinated manner with the cilia vibrating about a vertical axis and those on the same strip beating in opposite directions. The ciliary beating of cells in both culture systems was arrested instantly upon exposure to 0.1% w/v chlorocresol. The cilio-toxic effect of chlorocresol was reversible only at a concentration of 0.005% w/v. This reversibility was complete for cells in suspension and partial for cells in monolayer culture systems.

The cilio-toxicity of diphenhydramine was concentration dependent, slower in onset than with chlorocresol and more severe in the suspension culture than in the monolayer culture system. In the suspension culture system, incubating the cells with 0.1% w/v diphenhydramine for 15 and 30 minutes, respectively, resulted in $-50 \pm 4\%$ and $-69 \pm 3\%$ reduction of CBF. In the monolayer culture system, CBF decreased by $-45 \pm 7\%$ and $-41 \pm 5\%$ upon perfusion with 0.1% w/v diphenhydramine for 15 minutes and 30 minutes, respectively. The CBF decrease observed in the monolayer culture system was stable with time, while the decrease seen in the suspension culture was unstable. After the initial exposure of cells in the suspension culture system to diphenhydramine for 15 minutes, CBF decreased further by 19% on incubation for 30 minutes. However, CBF returned to baseline after washing the cells with DMEM-F12 1/1. Irreversible ciliostasis occurred within two minutes in both culture systems upon exposing the cells to 1.0% w/v diphenhydramine.

Effect of pH on Ciliary Beat Frequency (CBF)

The effect of pH on CBF of the monolayer and suspension culture systems is illustrated in Fig. 1. The CBF of cells in the suspension culture system appeared to be stable between pH 4.0 and 10.0. In the monolayer culture system, CBF was stable only between pH 6.5–8.0; at pH 4.0 a significant decrease ($-49 \pm 5\%$, $p < 0.0001$), was observed, while an increase ($+31 \pm 5\%$, $p < 0.0001$) occurred at pH 10.0. At a pH below 4.0 or above 11.0, irreversible ciliostasis occurred in both monolayer and suspension culture systems. A pH of 11.0 was too toxic with time to the cultured cells; however, an initial increase in CBF prior to morphological changes and cell death was observed. This increase was seen only in the first two to five minutes of CBF measurement; subsequently, CBF decreased with time. Instant ciliostasis occurred at an extreme pH of 2.0 and 12.0.

Additional controls of the 'waste' solution pH to exclude major pH shifts during the experiment revealed minor pH shifts towards neutral values from both basic and acidic conditions (data not shown). These pH shifts never exceeded 0.5, but were statistically significant for all pH values investigated except for pH 4.0 and 7.0.

DISCUSSION

The effects of pharmaceutical compounds on mucociliary clearance may be assessed in various models with a number of methods. In general, *in vivo* animal models give more reliable results of the effect of these compounds on ciliary activity in comparison to *in vitro* methods. This is partly due to the fact that in the *in vivo* methods the cilio-toxicity of compounds is attenuated by mucociliary clearance and mucus dilution. However, *in vivo* measurements in animals have a number of disadvantages: species differences, differences in anatomy of the nasal cavity compared to the human nose, high variability requiring large numbers of animals and difficulties in interpreting results that are influenced by many unknown factors (5). In addition, whole animal studies are not suitable for the large number of screening studies required during drug development (6). Consequently, *in vitro* determination of the effect of pharmaceutical compounds on ciliary beat frequency remains a valuable tool for assessing the influence of compounds on mucociliary transport. The effects of pharmaceutical compounds on ciliary activity has been the subject of extensive research and present knowledge originates mainly from investigations performed on explants from the upper airways (7). The duration of exposure time for such investigations is however limited because of degeneration of ciliary activity.

To overcome these problems, we decided to use cells of human origin, cultured with techniques that make short and long term exposure possible, namely the monolayer and the sequential monolayer-suspension culture techniques. The stability, reliability, reproducibility and relevance of CBF measured in these systems have already been demonstrated (1,4).

This study revealed the possibility of using cultured human nasal epithelial cells as models to test the effects of pharmaceutical compounds on ciliary activity. A comprehensive and comparative overview of the results obtained for reference cilio-inhibitory/stimulatory compounds is given in Table I.

Similar results were obtained for the same compound in both culture systems. The results were comparable with data in the literature. Only the acceleration of CBF by 18–26% in the cultured human nasal epithelial cells by glycocholate contradicted the mild decrease of CBF (10.0%) reported for this compound in human adenoid tissue (8). Though there are no literature data supporting the cilio-stimulation we observed with glycocholate in cultured human nasal epithelial cells, this compound was chosen as an in-house reference compound based on its consistency in increasing ciliary beating (at the concentrations and time exposure investigated). Secondly, glycocholate was used as a reference cilio-stimulatory compound to highlight the fact that glycocholate may indeed be safe for nasal drug delivery. In an *in vitro* study using rabbit trachea, it was reported that glycocholate had no significant effect on CBF at a concentration of 20 mM (9).

Isoprenaline reversibly increased the CBF of cells in both culture systems by 14–17%. The degree of cilio-stimulation by isoprenaline in both culture systems was lower than 28–30% CBF increase reported for human adenoid tissue (10,11).

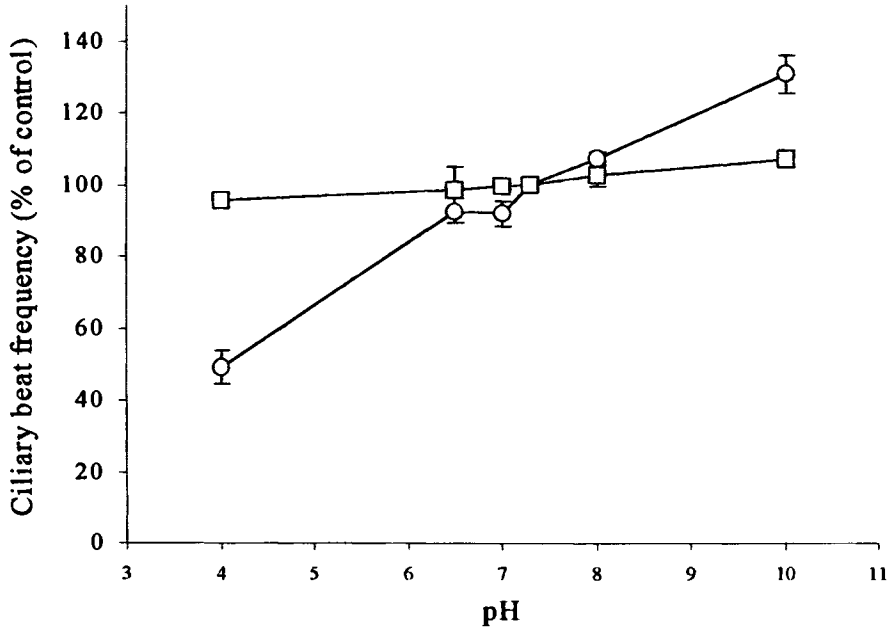

Fig. 1. Effect of pH on the ciliary beat frequency (CBF) of human nasal epithelial cells in the monolayer and suspension culture systems. Each point represents percentage CBF of treated group relative to CBF value of the control group ± S.E.M (n = 30 cells). —○— Monolayer culture system, —□— suspension culture system.

The cilio-toxicity and its reversibility of chlorocresol and diphenhydramine were concentration dependent. Similar results have been reported in chicken embryo trachea, rat and guinea pig tracheal explants (12,13,14). According to Van de Donk *et al.* (1980) (12), the reversibility of cilio-toxicity of chlorocresol (0.05%) was dependent on exposure time. At twice this concentration (0.1% w/v), we observed irreversible ciliostasis in both monolayer and suspension culture systems. These observations point to the fact that the main determinants of cilio-toxicity of chlorocresol and its reversibility are concentration and duration

Table I: Effects of Reference Compounds on Ciliary Beat Frequency (CBF) of the Monolayer and Suspension Culture Systems

	Monolayer culture system				Suspension culture system		
Compounds	Duration of exposure	Percentage CBF change ± SEM	Reversibility	Monolayer vs. suspension	Duration of exposure	Percentage CBF change ± SEM	Reversibility
Glycocholate							
(i) 0.005%	5 min.	4 ± ↓	No effect		5 min.	4 ± 2↑	No effect
	20 min.	7 ± 3↓	No effect	≅	20 min.	0	No effect
(ii) 0.5%	5 min.	26 ± 2↑*			5 min.	18 ± 3↑*	
	20 min.	25 ± 2↑*	Reversible	>	20 min.	13 ± 4↑*	Reversible
Isoprenaline							
(i) 10^{-5} M	30 min.	10 ± 2↑*			30 min	13 ± 3↑**	
	45 min.	9 ± 2↑*	Irreversible	≅	45 min.	11 ± 3↑**	Irreversible
(ii) 10^{-3} M	30 min.	11 ± 2↑*			30 min.	17 ± 8↑**	
	45 min.	10 ± 4↑*	Irreversible	<	45 min.	9 ± 3↑**	Irreversible
Chlorocresol							
(i) 0.005%	5 min.	46 ± 3↓*	Partially		5 min.	33 ± 2↓*	
	20 min.	51 ± 3↓*	reversible	>	20 min.	34 ± 2↓*	Reversible
(ii) 0.1%	less than 1 min.	100 ↓*	Irreversible	≅	less than 1 min.	100↓*	Irreversible
Diphenhydramine							
(i) 0.1%	15 min.	45 ± 7↓*			15 min.	50 ± 4↓*	
	30 min.	41 ± 5↓*	Reversible	<	30 min.	69 ± 3↓*	Reversible
(ii) 1.0%	less than 2 min.	100↓*	Irreversible	≅	less than 2 min.	100↓*	Irreversible

Note: ↑ = CBF increase, ↓ = CBF decrease, ≅ = similar effect in both cultures, > = greater effect in monolayer culture, < = less effect in monolayer culture, * = $p < 0.001$), ** = $p < 0.05$.

of exposure. Similarly, 0.1% w/v diphenhydramine caused severe (45–69%), but reversible cilio-toxicity in both culture systems. The cilio-stasis that resulted upon exposing the cells to a higher concentration of diphenhydramine (1.0% w/v) confirmed the high cilio-toxicity of this compound reported in rat tracheal and chicken embryo cilia (15,16).

Within physiological range, the effect of pH on the ciliary activity of the cultured cells was comparable and reproducible in both culture systems. This study revealed that in cultured human nasal epithelial cells, alkaline pH appears to be more favorable to ciliary activity than an acidic environment. The suspension culture system was more resilient to pH changes in both acidic and basic regions. The higher stability of CBF in the suspension culture in comparison to the monolayer culture may be explained by the absence of basolateral membrane exposure to test solutions in the suspension culture system. In the suspension system, cells are tightly clustered as spheroid aggregates with tight junctions. A pH range of 6.5–10.0 has been reported to be ideal for ciliary beating in human nasal biopsies, rat tracheal explants and chicken embryo (17,18,19). In ciliated epithelium obtained from lower airways (bronchi and bronchioles), CBF stability was reported between pH 7.5–10.5 for bronchi and 5.5–10.5 for bronchioles (20). The results of our study agreed with above published reports. However, significant decrease in CBF within alkaline pH range was reported in rat and chicken embryo cilia (19). These differences in threshold pH values may represent variations due to the method of CBF determination rather than species characteristics (20).

On a comparative basis, the sequential monolayer-suspension culture holds greater prospects than the monolayer culture system for routine cilio-toxicity screening of pharmaceutical compounds. This is based on the fact that ciliary activity may degenerate rapidly with time in the monolayer culture system, while it can be maintained for a long period of time in the sequential monolayer-suspension culture. In addition, CBF studies involving pharmaceutical compounds are easier to conduct using suspension culture system as cumbersome manipulations involving perfusion (peculiar to the monolayer culture system) are avoided. Also, cells in the suspension culture exist as spheroids and are easier to visualize under the microscope. In contrast, the nature of cell topography in the monolayer culture system is a disadvantage with respect to measuring the CBF of the same cells after drug exposure.

The sequential monolayer-suspension culture system offers the advantage of ensuring constant supply of ciliated cells needed for large numbers of studies during nasal drug development. This is important considering the growing interest in nasal drug administration for systemic purposes.

CONCLUSIONS

As the results obtained in this study compare favorably well with literature data, it can be concluded that the monolayer and sequential monolayer-suspension cell culture systems are suitable for screening the effects of pharmaceutical compounds on ciliary beating, especially for screening cilio-toxicity. As the sequential monolayer-suspension culture offers the advantage that ciliary activity can be preserved for as long as 6 months, this culture method appears to be very promising.

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