Simultaneous Measurement of Ciliary Beating and Intracellular Calcium

Alon Korngreen and Zvi Priel
Department of Chemistry, Ben-Gurion University of the Negev, Beer-Sheva 84105 Israel

ABSTRACT A novel system for measuring, simultaneously, ciliary beating and intracellular free calcium is presented. The advantages and dynamic nature of the system are demonstrated by measuring the effects of the calcium ionophore Ionomycin and of extracellular ATP on ciliated rabbit trachea. The results are discussed with regard to the ciliary and calcium stimulation.

INTRODUCTION

Cilia, those small organelles protruding from the cell surface, have been the issue of intense research for most of this century. The main function of cilia is biological transport either of a single cell through water (Gibbons, 1981) or of a mucus layer over the cells (Sleigh et al., 1988). To fulfill these tasks, cilia beat synchronously, creating a wave-like pattern on the cell surface called the metachronal wave. Water-propelling cilia, which are found in ciliated protozoa and molluscs, are relatively long (10–30 μM), contrary to mucus-transporting cilia, which are shorter (5–7 μM) and more densely packed. These differences make mucus-transporting cilia harder to investigate. Ciliary systems are dynamic ones, responding with a change in their beating parameters to a variety of stimuli, mechanical (Dirksen and Sanderson, 1989; Sanderson et al., 1990), chemical (Aiello et al., 1991; Weiss et al., 1992; Wong and Yeates, 1992), electrical (Mogami et al., 1990), and hormonal (Villalon et al., 1989). These changes of the ciliary beat patterns, which can be followed by relatively simple means, add a lot of information on the events taking place inside the cell. Therefore, cilia can perform for us the function of an indicator for the activities going on inside the cell without the need to introduce an artificial indicator into the delicate environment of the cell.

One of the cellular events that is directly related to a change in ciliary beating is the level of intracellular calcium concentration. It was shown (Naitoh and Kaneko, 1972) in permeabilized paramecium that there is a correlation between the calcium concentration and the ciliary beat parameters. Calcium was also shown to activate directly the beating of isolated cilia (Tamm, 1988, 1989). In mucus-transporting cilia, the role of intracellular calcium concentration was investigated more intensely over the last few years (Dirksen and Sanderson, 1989; Sanderson et al., 1990; Villalon et al., 1989; Weiss et al., 1992) since the introduction of the novel intracellular calcium indicator fura-2 (Tsien et al., 1985).

The calcium second messenger signaling system is a highly dynamic one. Over the last decade, a paucity of data was revealed regarding the functional role of cytosolic calcium in the living cell. Intracellular free calcium is an important link in the chain of signal transduction from the stimuli on the cell surface to the cellular response. The simplicity that was at first attributed to the calcium messenger system has gradually been replaced by the realization that this system has complex behavior both in time and in space. It has been argued (Rasmussen and Rasmussen, 1990) that after the initial rise in cytosolic free calcium concentration ($[Ca^{2+}]_i$), usually caused by release of calcium from the internal calcium stores, there is a calcium influx resulting in a rise of $[Ca^{2+}]_i$ in small submembranous domains, thus prolonging the duration of the cellular response without risking cell toxification by a high concentration of calcium. These submembranous domains are by nature hard to detect because of the inability of the current technique to visualize the calcium concentration at such small volumes.

Ciliary cells are inherently suitable for detecting changes in the calcium concentration near the plasma membrane. Because the cilia are closely covered by the plasma membrane, any change in the calcium concentration that occurs there will effect the ciliary beating. Another aspect of both ciliary beating and intracellular calcium is their ability to produce oscillations both in calcium and in frequency as has been shown lately on sheep trachea (Salathe and Bookman, 1993). Because the changes in ciliary beating and in calcium concentration have great variability and much averaging is needed to discern trends in each of them, it is not enough to measure 1) the response to a given stimulation, 2) the ciliary beat frequency, and 3) the change in intracellular calcium concentration separately. Therefore, we developed a novel apparatus that enables us for the first time to follow the changes in ciliary beat frequency and in intracellular calcium concentration simultaneously and on the same ciliary cell.

MATERIALS AND METHODS

Chemicals and solutions

All chemicals were obtained from Merck (Dramstadt, Germany) unless stated otherwise. Both the pentapotassium salt (K_5fura-2) and the acetoxymethyl ester (fura-2/AM) forms of the calcium indicator fura-2 were obtained from Molecular Probes (Eugene, OR). The dye was stored as a solid at −18°C, and fresh solutions were made immediately before the experiment

Received for publication 25 October 1993 and in final form 25 February 1994.

Address reprint requests to Zvi Priel, Department of Chemistry, Ben-Gurion University of Negev, P.O.B. 653, 84105 Beer-Sheva, Israel. Tel.: 972-7-461184; Fax: 972-7-274359; E-mail: alon@bgumail.bgu.ac.il.

0006-3495/94/07/377/04 $2.00

in dimethyl sulfoxide (DMSO). Ionomycin was obtained from Calbiochem (Lucerne, Switzerland); it was kept as a 5 mM solution in 100% ethanol at −18°C, and aliquots were diluted in Ringer solution before the experiment. ATP sodium salt was obtained from Sigma Chemical Co. (St. Louis, MO). The standard Ringer solution was composed of (in mM) 140 NaCl, 1 Na_2PO_4, 6 KCl, 1.3 $CaCl_2$, 1 $MgCl_2$, 5 D-glucose, and 3 HEPES. The pH of the Ringer solution was set to 7.4 with NaOH and HCl. The external calibration solutions for fura-2 were composed of (in mM) 115 KCl, 20 NaCl, 5 $MgCl_2$, 5 D-glucose, 5 HEPES, 10 EGTA, and 1 μM K_5fura-2. Solutions with low concentrations of $CaCl_2$ were prepared with 0.5 mM EGTA utilizing a computer program to calculate the free calcium concentration of these solutions (Fabiato, 1989).

Tissue culture

Adult white rabbits were killed with CO_2. The trachea was removed, the ciliary epithelium was pealed off and cut into pieces of approximately 1 mm in diameter. The pieces were placed on glass cover slips that were previously incubated in growth media. The glass cover slips were then placed in plastic Petri dishes (Nunc, Denmark) with a few drops of RPMI-1640 growth media and incubated at 37°C and 5% CO_2. After 24-h growth, media were added to a total volume of 0.5 ml and were replaced every 2 days. The cells were used after 1 week in culture.

Simultaneous measurement of intracellular calcium and ciliary beating

The simultaneous measuring apparatus was built around a Ziss Axioskope fluorescence microscope, displayed schematically in Fig. 1. Excitation light for the fura-2 was received from a 150 W Xenon lamp (Oriel Corp., Stamford, CT) filtered by interference filters (340 and 380 nm, full-width at half maximum = 10 nm; Oriel) mounted on a filter wheel that rotated, at a fixed frequency, in the range of 25–30 Hz. The rotation speed was monitored by a photo diode bridge mounted on the rim of the wheel. The light was attenuated by an iris diaphragm to reduce the illuminated field to the size of three to four cells, reflected by a dichroic mirror and focused on the culture by an X40 objective (Zeiss Acroplan, NA = 0.6). The emitted light was directed by a prism to the photo eyepiece, filtered by a 510 nm interference filter (Oriel) and passed into a photon-counting head (Hamamatsu, Japan) mounted directly on the photo eyepiece. The photon-counting head produced a standard logical pulse (TTL), which was counted by a computer counter board inserted into a IBM PC 486 compatible computer. The 340/380 fluorescent ratio was calculated for every turn of the filter wheel and averaged to give a data point for every 2 s with a delay of 0.1 s due to computation time of the fluorescent ratio and the ciliary beating frequency. We are able to measure intracellular calcium from a smaller area, down to a single cell on the culture by closing an aperture positioned near the photon-counting head. Measurements performed at several aperture sizes produced the same results. We chose to measure the calcium concentration from an area of three to four cells to improve the signal-to-noise ratio.

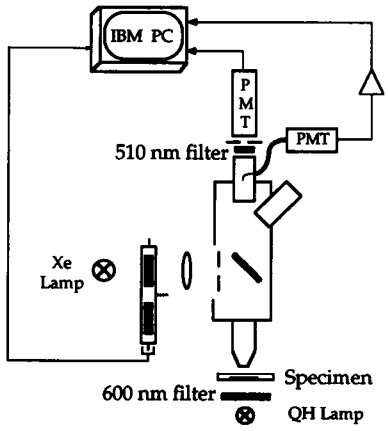

FIGURE 1 Schematic drawing of the simultaneous measurement system. See text for details.

To measure the ciliary beat frequency, the cells were transilluminated with light from a Quartz Halogen lamp that was filtered by a 600 nm interference filter (full-width at half-maximum = 10 nm; Oriel). This wavelength of light was chosen because it does not participate in either the excitation or emission processes of fura-2. After passing through the sample and the objective, this light was also directed to the photo eyepiece, which contained at the focal plane, as illustrated in Fig. 1, a 50 μm diameter optic fiber (EG&G Gamma Scientific). This fiber, at a magnification of 40, collects light from a surface of equivalent diameter of 1.25 μm on the tissue culture (Eshel and Priel, 1987). This diameter enables us to measure ciliary beat frequency from part of the cell. The light was passed by an optic fiber bundle to a photomultiplier (PMT), and the resulting electrical signal was amplified and low pass-filtered to reduce noise. Intervals of 2 s, at which the calcium concentration was also monitored, were digitized at a sampling rate of 1024 Hz, and a Fast Fourier Transform (FFT) was applied to segments of 1 s with an overlap of a quarter of a second, and the resulting power spectra were averaged. Measurements of ciliary beat frequency, which were performed on several adjacent places on the same cell and on neighboring cells, showed complete correlation of the beat frequency. Moreover, simultaneous measurements of ciliary beat frequency from three adjacent cells (Gheber and Priel, 1994) showed that after addition of extracellular ATP the ciliary beat frequency rises at the same time over the entire measured area.

Loading of cells with fura-2

The growth media were removed from the Petri dishes containing the cultures. A solution of 5 μM fura-2/AM in 1 ml of RPMI-1640 was added, and the culture was incubated for 60 min at 37°C in a rotating water bath. The culture was washed with Ringer solution for 50–60 min at room temperature. This loading procedure is similar to that mentioned in the literature for loading fura-2 into rabbit tracheal ciliary cells (Boitano et al., 1992; Sanderson et al., 1990). The cover slips with the cells were kept in a custom-made perfusion cell, which was kept at 37°C by a water-heated plate for the duration of the experiment. This cell allowed rapid exchange of the Ringer solution without changing the flow rate of the solution, which might mechanically stimulate the cilia. Experiments were carried out only on evenly fluorescent cells with no bright spots. In some experiments, the total concentration of the dye was monitored at 360 nm, a wavelength at which fura-2 is insensitive to calcium concentration, and a decrease in intensity of a few percent was observed after half an hour of illumination. This decrease can be the result of minor photobleaching or of leakage of the dye from the cell. This decrease in intensity was small and did not affect the measurements.

Calibration of calcium concentration

A calibration curve of calcium concentration was created by titration of an external calibration solution with a solution with the same composition containing also 10 mM $CaCl_2$ (Grynkiewicz et al., 1985; Tsien et al., 1985). To stretch the usefulness of the calibration curve to higher calcium concentrations and to avoid the inaccuracies resulting from using the fura-2 dissociation constant from the literature, $[Ca^{2+}]_i$ was calculated directly from the calibration curve by interpolation using a table look-up algorithm. The minimal and maximal values of the 340/380 fluorescent ratio, R_{min} and R_{max} were 0.49 ± 0.02 and 14 ± 1, respectively. These values agree with the R_{max}/R_{min} > 25 ratio associated with fura-2.

RESULTS AND DISCUSSION

The results draw a complex picture of the calcium signaling and ciliary beating and their interrelations in rabbit tracheal cells and emphasize the unique advantages and dynamic

scope of the measuring system. It was shown in permeabilized paramecium (Naitoh and Kaneko, 1972) that ciliary beating is directly correlated to calcium concentration. Therefore, to bypass the signaling pathways and apply calcium directly to the cilia, we applied the calcium ionophore Ionomycin to the culture. After the admission of 5 μM Ionomycin, $[Ca^{2+}]_i$ begins to rise slowly from a resting value of 270 $\pm$ 20 reaching a value of 300 nM after 10 s. At that point in time, there is a rapid rise in ciliary beating from a resting value of 10 $\pm$ 2 Hz to a steady value of 27 Hz as displayed in Fig. 2 *A*. There is an initial rapid rise in the beat frequency to 23 Hz over the first 2 s after the stimulation followed by slower rise to 27 Hz lasting 4 more s, which is easily discernible from the enlargement of part of the experimental curve displayed in Fig. 2 *B*. During this time, there is only a minor rise in $[Ca^{2+}]_i$ from 300 to 350 nM. Only 50 s after the admission of Ionomycin, the level of $[Ca^{2+}]_i$ reaches a maximal value of 570 nM. Three minutes after the stimulation, the calcium concentration has returned almost to its value before the stimulation, whereas the ciliary beat frequency decays to a steady level of 22 $\pm$ 1 Hz. These results show that the cilia were activated by the calcium diffusing into the cell. Because the cilia are closely covered by the cell membrane while the volume of the cytoplasm in the cilia is relatively small, the calcium diffusing into the cell reaches the cilia first and only after a substantial amount of time floods the entire cell, thus causing a maximal response of the calcium indicator fura-2.

We have shown that extracellular ATP, a potent neurotransmitter, causes a complex behavior of intracellular calcium in human ciliary cells (A. Korngreen and Z. Priel, unpublished data), activating both a release of calcium from internal stores and an ensuing influx of calcium. Applying 50 μM extracellular ATP to ciliary rabbit tracheal cells results in a rise of ciliary beating from 13 to 24 Hz and of intracellular calcium from 150 to 440 nM, as shown in Fig. 3 *A* and enlarged in Fig. 3 *B*. As in the case of Ionomycin, there is an initial rapid rise in the ciliary beat frequency over the

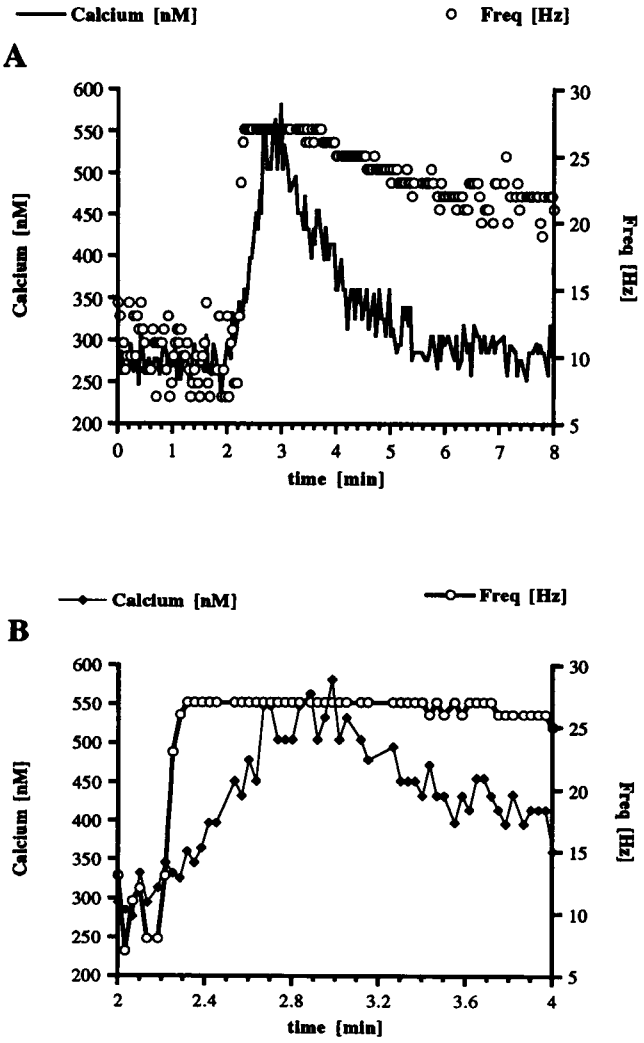

FIGURE 2 Typical effect of 5 μM Ionomycin on ciliary beating and intracellular calcium concentration (*A*) and an enlargement of the part of the experiment directly after the stimulation (*B*).

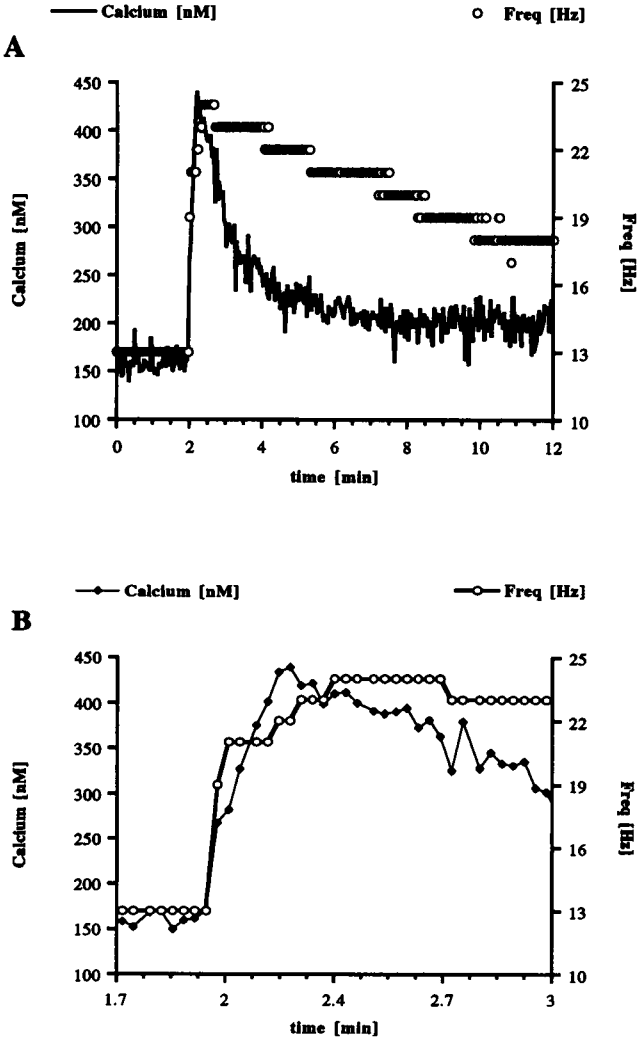

FIGURE 3 Typical effect of 50 μM extracellular ATP on ciliary beating and intracellular calcium concentration (*A*) and an enlargement of the part of the experiment directly after the stimulation (*B*).

first 2 s after the stimulation followed by a slower rise, lasting 20 s to the maximal value. The rise in $[Ca^{2+}]_i$ displays a similar behavior; there is an initial rapid rise in the calcium concentration over the first 2 s followed by a slower one lasting only 10 s, which is 2 times faster than the rise time of the ciliary beat frequency. The calcium concentration already begins to decay from its maximal value when the ciliary beat frequency reaches its maximal value. The calcium concentration decays 3 min after the stimulation to a higher level than the concentration before the stimulation, and the ciliary beat frequency displays a constant decay (the step-like form of the graph is due to the discrete nature of the Fourier transform), not reaching the initial value even 10 min after the stimulation. Washing the ATP away from the external solution results in a decrease in $[Ca^{2+}]_i$ and in ciliary beat frequency to their values before the stimulation (data not shown). Moreover, after the depletion of the intracellular calcium stores with Thapsigargin, in a calcium free medium, extracellular ATP had no effect on either intracellular calcium and ciliary beat frequency (data not shown).

The behavior of the ciliary cells to the stimulation with ATP can be easily explained in view of the experiment with Ionomycin. ATP triggers a chain of events, starting with a plasma membrane receptor, continuing with release of calcium from internal stores, as was shown for mechanical stimulation of rabbit ciliary cells (Sanderson et al., 1990), causing a rise in calcium inside the cell, which stimulates the cilia. Because the calcium stores are stationed inside the cell, it is probable that calcium has to diffuse over the whole length of the cilium to activate fully all of the contractile units in it. It was shown in isolated cilia (Tamm, 1988, 1989) that the axoneme response to an increase in calcium concentration is an activation of beating. Therefore, the calcium concentration has already reached its maximal value inside the cell, whereas the cilia have not been fully activated. It is also clear from the experiments with the two stimulants that there is a two-stage behavior of the ciliary stimulation. There is an initial rapid rise in the ciliary beat frequency followed by a slower one to the maximal value.

We believe that these experiments shed light on the ciliary stimulation mechanism and on the calcium messenger system. We have shown that by measuring the output of these two systems simultaneously we can gain better understanding of each one of these systems than by measuring each one separately, especially because our results show that the ciliary beating and the $[Ca^{2+}]_i$ do not correlate fully. At the present level of technology, it is impossible to monitor the calcium concentration at very small domains in the cell. The use of cilia as a probe for those can provide valuable information on the existence of high levels of calcium near the plasma membrane. Moreover, the events leading from the rise in $[Ca^{2+}]_i$ to the stimulation of the cilia can now be investigated by looking at both the cause and the effect in real time. To the best of our knowledge, this is the first time that ciliary beating and intracellular calcium concentration were measured simultaneously.

REFERENCES

Aiello, E., J. Kennedy, and C. Hernandez. 1991. Stimulation of frog ciliated cells in culture by acetylcholine and Substance P. *Comp. Biochem. Physiol.* 99:497–506.

Boitano, S., E. R. Dirksen, and M. J. Sanderson. 1992. Intracellular propagation of calcium waves mediated by inositol triphosphate. *Science.* 258: 292–295.

Dirksen, E. R., and M. J. Sanderson. 1989. Regulation of ciliary activity in the mammalian respiratory tract. *Biorheology.* 27:533–545.

Eshel, D., and Z. Priel. 1987. Characterization of metachronal wave of beating cilia on frog's palate epithelium in tissue culture. *J. Physiol.* 388:1–8.

Fabiato, A. 1989. Computer programs for calculating total from specific free or free from specific total ionic concentrations in aqueous solutions containing multiple materials and ligands. *Methods Enzymol.* 157:378–417.

Gheber, L., and Z. Priel. 1994. Metachronal activity of cultured mocociliary epithelium under normal and stimulated conditions. *Cell Motil. Cytoskel.* In Press.

Gibbons, I. R. 1981. Cilia and flagella of eukaryotes. *J. Cell Biol.* 91: 107s–124s.

Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca^{2+} indicators with improved fluorescence properties. *J. Biol. Chem.* 260:3440–3450.

Mogami, Y., J. Pernberg, and H. Machemer. 1990. Messenger role of calcium in ciliary electromotor: a reassessment. *Cell Calcium.* 11:665–673.

Naitoh, Y., and H. Kaneko. 1972. Reactivated Triton-extracted models of paramecium: modification of ciliary movement by calcium ions. *Science.* 176:523–524.

Rasmussen, H., and J. E. Rasmussen. 1990. Calcium as intracellular messenger: From simplicity to complexity. *Curr. Top. Cell. Regul.* 31:1–109.

Salathe, M., and R. J. Bookman. 1993. Single cell measurement of ciliary beat frequency and intracellular calcium in tracheal epithelial cells. *Biophys. J.* 64:264*a*. (Abstr.)

Sanderson, M. J., A. C. Charlest, and E. R. Dirksen. 1990. Mechanical stimulation and intercellular communication increases intracellular Ca^{2+} in epithelial cells. *Cell. Regul.* 1:585–596.

Sleigh, M. A., J. R. Blake, and N. Liron. 1988. The propulsion of mucus by cilia. *Am. Rev. Respir. Dis.* 137:726–741.

Tamm, S. L. 1988. Calcium activation of macrocilia in the ctenophore *Beroe. J. Comp. Physiol. [A]* 163:23–31.

Tamm, S. L. 1989. Control of reactivation and Microtubule sliding by calcium, strontium, and barium in detergent-extracted macrocilia of *Beroe. Cell Motil. Cytoskel.* 12:104–112.

Tsien, R. Y., T. J. Rink, and M. Poenie. 1985. Measurement of cytosolic free Ca^{2+} in individual small cells using fluorescence microscopy with dual excitation wavelengths. *Cell Calcium.* 6:145–157.

Villalon, M., T. R. Hinds, and P. Verdugo. 1989. Stimulus-response coupling in mammalian ciliated cells. Demonstration of two mechanisms of control for cytosolyc $[Ca^{2+}]$. *Biophys. J.* 56:1255–1258.

Weiss, T., L. Gehber, V. Shoshan-Bramatz, and Z. Priel. 1992. Possible mechanism of ciliary stimulation by extracellular ATP: involvement of calcium-dependent potassium channels and exogenous Ca^{2+}. *J. Membr. Biol.* 127:185–193.

Wong, L. B., and D. B. Yeates. 1992. Luminal purinergic regulatory mechanisms of tracheal ciliary beat frequency. *Am. J. Respir. Cell. Mol. Biol.* 7:447–454.