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Coupling of $[Ca^{2+}]_i$ and ciliary beating in cultured tracheal epithelial cells

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SUMMARY

The molecular mechanisms responsible for the regulation of ciliary beating frequency (CBF) are only partially characterized. To determine whether elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) can cause an increase in CBF, we measured CBF and Ca^{2+} in single cells. Ovine tracheal epithelial cells, obtained by dissociation with protease, were grown in primary culture for 1 to 28 days in a mucus-free system. CBF of a single cilium was measured by digital video phase-contrast microscopy and on-line Fourier-transform analysis. Changes in $[Ca^{2+}]_i$ from single cells were determined with fura-2, using ratio imaging video microscopy. Activation of a muscarinic pathway with 10 μM ACh (acetylcholine) increased $[Ca^{2+}]_i$ from 53±9 nM (mean ± s.e.m.) to 146±12 nM or to 264±51% above initial baseline. In the same cells, ACh increased CBF from a baseline of 7±0.5 Hz to 9±0.2 Hz or to 31±2.8% above baseline (n=14). The elevations of both $[Ca^{2+}]_i$ and CBF were transient and relaxed back to an elevated plateau (10/14 cells) as long as ACh was present. To elevate $[Ca^{2+}]_i$ by mechanisms independent of a G-protein-coupled receptor, we measured $[Ca^{2+}]_i$ and CBF of the same cells in extracellular solutions with either 0 Ca^{2+} (+ 1 mM EGTA) or 10 mM Ca^{2+}. Both signals rose and fell with similar kinetics in response to changing $[Ca^{2+}]_o$, suggesting that changes in $[Ca^{2+}]_i$ alone can modulate CBF. In a second independent manipulation, cells were treated with 1 μM thapsigargin, an irreversible inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase. Upon thapsigargin addition, 37 of 42 cells showed a transient $[Ca^{2+}]_i$ increase and, as measured in different experiments, 8 of 9 cells showed a transient increase in CBF. Interestingly, application of ACh after cells were treated with thapsigargin produced decreases in both $[Ca^{2+}]_i$ and CBF in 8/8 cells. Lastly, after 1-3 days in culture, addition of 10 μM ACh often produced $[Ca^{2+}]_i$ oscillations rather than transients in $[Ca^{2+}]_i$. Measurements of CBF in these cells showed frequency modulation of CBF with the same peak-to-peak time interval as the Ca^{2+} oscillation. These results show that: (1) CBF can be measured from a single cilium and monitored on-line to track changes; (2) CBF and $[Ca^{2+}]_i$ can be measured in the same single cell; (3) transient changes in $[Ca^{2+}]_i$ (induced by 4 different manipulations) are associated with kinetically similar changes in CBF; and (4) $[Ca^{2+}]_i$ oscillations are coupled to frequency modulation of ciliary beating. Taken together, these results provide strong evidence that $[Ca^{2+}]_i$ is a critical intracellular messenger in the regulation of CBF in mammalian tracheal epithelial cells.

Key words: cilium, calcium, acetylcholine, trachea, epithelium, video microscopy

INTRODUCTION

The mucociliary transport system of the tracheal epithelium is a major host defense mechanism of the lungs. It consists of a propulsive motor, namely beating cilia on the apical surface of the airway epithelium, and a carrier for foreign material, mucus secreted by goblet cells and submucosal glands. The adaptation of this transport system to different challenges is essential to maintain effective clearance in the airway. Despite the critical role of ciliary activity in this transport function, the cellular and molecular mechanisms responsible for regulating ciliary beating frequency (CBF) are still incompletely understood.

In unicellular organisms, widely used for the study of cilia, intracellular free calcium ($[Ca^{2+}]_i$) plays a crucial role with respect to the speed and direction of ciliary beating. In *Paramecium*, for instance, rising $[Ca^{2+}]_i$ slows CBF to the point where the beat direction is reversed (Naitoh and Kaneko, 1972). Mammalian cilia, on the other hand, always beat in the same direction with respect to the direction of their effective stroke and $[Ca^{2+}]_i$ cannot play this complex role (Satir and Sleigh, 1990). Studies on mammalian cilia have suggested that $[Ca^{2+}]_i$ might also be important in the regulation of CBF and it appears that increasing $[Ca^{2+}]_i$ increases CBF (Verdugo, 1980; Girard and Kennedy, 1986; Villalon et al., 1989; Di Benedetto et al., 1991; Lansley et al., 1992). In all these studies, however, the $[Ca^{2+}]_i$ and CBF signals were measured in different cells, thus making comparisons at the single cell level impossible. Furthermore, as described below, different ciliated cells on a coverslip can beat at different frequencies and the presence of nonciliated cells in the culture will contribute to the measured $[Ca^{2+}]_i$ responses. Therefore, data obtained from populations of cells (e.g., cuvette methods or ^{45}Ca flux measurements for $[Ca^{2+}]_i$ estimates and multi-cell

areas for CBF measurements; Girard and Kennedy, 1986; Di Benedetto et al., 1991) may be difficult to interpret and are unlikely to provide the best system for investigating the molecular mechanisms of CBF regulation.

To test the hypothesis that $[Ca^{2+}]_i$ is a critical messenger in the regulation of CBF, we developed a method for single cilium recording that enables us to measure CBF and $[Ca^{2+}]_i$ of the same cultured ciliated cell. In an effort to study its effects on CBF, we manipulated $[Ca^{2+}]_i$ in three different ways: (1) by stimulating a muscarinic receptor-coupled pathway (Wong et al., 1988; Mak et al., 1992) using acetylcholine (ACh); (2) by changing the concentration of extracellular Ca^{2+}; and (3) by releasing Ca^{2+} from intracellular stores using thapsigargin, an irreversible endoplasmic reticulum (ER) Ca^{2+}-ATPase inhibitor (Thastrup et al., 1990). Preliminary reports of these results have been presented at meetings of the Biophysical Society and the American Thoracic Society (Salathe and Bookman, 1993a,b; Salathe et al., 1994).

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle's medium (DMEM), Ham's nutrient F-12, and Hanks' balanced salt solution were purchased from Gibco Laboratories (Grand Island, New York); fura-2/AM (F-1225) and fura-2 K^+-salt (F-1200) from Molecular Probes (Eugene, Oregon); thapsigargin from Calbiochem (San Diego, California). All other chemicals were obtained from Sigma Chemicals (St Louis, Missouri). Thapsigargin was dissolved in dimethylsulfoxide (DMSO) as a stock solution of 1 mM and diluted into HBSS 1:1000 just prior to use.

Preparation of tracheal epithelial cultures

Primary cultures of sheep tracheal epithelial cells were prepared using an adaptation of published methods (Wu et al., 1985; van Scott et al., 1988; Zeitlin et al., 1988; Kondo et al., 1991). Segments of trachea 10-15 cm in length, were obtained from freshly killed ewes (30 mg/kg pentobarbital). The mucosa was dissected from the underlying cartilage under sterile conditions, rinsed with DMEM, and incubated in 0.05% protease (Sigma, type 14) in DMEM for 18 hours at 4˚C. To harvest cells, the mucosal strips were then vigorously shaken for 15 seconds at 21˚C and the protease digestion was stopped by adding fetal calf serum (FCS; Hyclone) to a final concentration of 5%. The cells were pelleted at 150 ***g*** for 45 seconds at 21˚C and twice washed with DMEM containing 5% FCS. Finally, the cells were resuspended in 50% DMEM, 50% Ham's nutrient F-12 containing 5% FCS, penicillin (100 i.u./ml), and streptomycin (100 μg/ml), and plated on collagen-coated glass coverslips (human placental collagen, type VI, Sigma) at a density of 0.5×10^6 cells/cm^2 in a minimal volume of 100 μl/cm^2. The cell viability as estimated by trypan blue exclusion was always >90%. Cells were grown in an incubator at 37˚C in 5% CO_2/95% air at 98% relative humidity. At 24 hours after plating, the culture medium was replaced with growth factor medium (50% DMEM, 50% Ham's nutrient F-12 containing insulin (10 μg/ml), transferrin (5 μg/ml), hydrocortisone (0.36 μg/ml), triiodothyronine (20 ng/ml), endothelial cell growth supplement (7.5 μg/ml), penicillin 100 i.u./ml, and streptomycin 100 μg/ml). The medium was exchanged every other day. Cultures were used 1-28 days after plating. Immunostaining of the cultures showed that >95% of the cells were positive for cytokeratin, no cells were positive for α-actin (a smooth muscle cell marker) or rhodamine-coupled, acetylated LDL (an endothelial cell marker). Periodic acid/Schiff (PAS) staining revealed that the cells did not contain PAS positive granules, indicative of mucus-free cultures.

Measurement of CBF

Single cilium recording of beat frequency was achieved by a photometric method that we developed. Coverslips with cultured cells were placed into an open Dvorak-Stotler chamber (Nicholson Precision Instruments, Bethesda, MD) and mounted onto the stage of a Nikon Diaphot inverted microscope (Nikon, Inc., Garden City, NY) at 21-23˚C. The cells were imaged with phase-contrast optics and a 100×, 1.3 NA Nikon CF Fluor DL oil immersion objective providing a final optical gain of 1,000×. This level of magnification included ~10 cells per field and permitted us to clearly visualize individual cilia and basal bodies. Transillumination was provided by an IR-filtered 100 W tungsten source. A multi-image module, (Nikon, Inc.) attached to the side port of the microscope, held two video cameras. For on-line CBF measurements, the light path was directed to a Hitachi video camera (Model HV-720U). Video signals were digitized and processed with an IC300 Image Processing Workstation (Inovision Corp., Durham, NC) and a SparcStation IPC (Sun Microsystems Computer Corp., Mountain View, CA). Using software that we developed, single pixels (180 nm by 180 nm) from the live, digitized, contrast-enhanced video image were selected, and the light intensity of the selected pixels was recorded on a frame by frame basis at 30 Hz. Alternatively, the magnitude spectrum from a fast Fourier transform (FFT) of the pixel's intensity was computed on-line and displayed on the monitor. Typically, 128 samples were used to compute the FFT. With RS-170 video timing (30 frames per second; Inoue, 1986), the bandwidth is 15 Hz, the frequency resolution of the system is 15/(128/2) = 0.23 Hz, and the acquisition of 128 samples takes 4.26 seconds. With the time required to sample the video signal, compute the FFT, update the screen displays, and write the spectrum to disk, a new FFT sample was acquired every ~6 seconds. FFT time series data were imported into Spyglass Transform (Spyglass Inc., Champaign, IL) where they were interpolated, low-pass filtered and viewed as two-dimensional images using a gray scale or color look-up table. Some FFT series were peak-extracted and the data used for plot graphs.

In practice, the microscope objective was focused just above the basal body of the cilium. This plane of focus was chosen because the complicated movement of a single cilium is reduced to a main backward and forward movement at the base of the cilium (see Fig. 1A). To confirm that the chosen pixel was giving a clear frequency peak in the magnitude spectrum, we computed a single FFT from 128 samples. If there was no single clear peak, another pixel or plane of focus was tried until a clear spectrum was obtained. During preliminary experiments, we encountered two problems with this single-pixel based method. First, the pattern of movement of an individual cilium often shifted from the plane of focus during the course of a 20-60 minute recording. This necessitated stopping the recording for ~20-40 seconds to adjust the focus and then resuming the recording. Second, small changes in the shape of the cell shifted the basal body away from the sampled pixel. Thus we found that recording the activity of only a single pixel could fail to track the optimal signal. To solve this latter problem, we chose to monitor the nine pixels in a 3 by 3 box (~0.5 μm^2) centered around the selected pixel. By tracking nine individual FFT spectra and selecting from each set of nine the one with the greatest peak value, we constructed a composite CBF time course that was able to track the cilium during the course of a 60-100 minute experiment.

Measurement of $[Ca^{2+}]_i$

Incubation protocol for dye-loading

Coverslips were rinsed several times with Hanks' Balanced Salt Solution buffered with 10 mM HEPES, pH 7.4 (called HBSS), and then loaded at room temperature on a rocking table with a sonicated solution containing 4 μM fura-2/AM, 2.5% FCS, in HBSS for 45 minutes. The dishes were washed several times with HBSS and the coverslips were then mounted into an open Dvorak-Stotler chamber with a working volume of ~0.5 ml.

Imaging hardware and software

The fluorescence excitation system is one of our own design (F. Horrigan, M. Cannell, and J. Bookman, unpublished data) and permits the output of a 100 W mercury lamp to be rapidly switched to one of six interference filters in less than 1 ms. With fura-2 as the $[Ca^{2+}]_i$ indicator, 10 nm wide filters centered on 340 nm and 380 nm were used (Chroma Technology Corp., Brattleboro, VT). The fluorescence image of the emitted light (600 nm > x > 510 nm) was visualized either with a 40×, 1.3 NA or with a 100×, 1.3 NA oil immersion objective (CF Fluor DL series, Nikon, Inc.), directed out the side port of the microscope, through the multi-image module, to a KS1381 micro-channel plate image intensifier (Opelco Inc., Washington, DC), coupled to a Hamamatsu 2400 CCD camera. Individual cells were identified as regions of interest (ROIs). As many as 64 ROIs were followed simultaneously, thereby permitting us to track either many cells or subcellular regions. A total of 8-32 video frames were averaged at each of the two excitation wavelengths and the ratio within each ROI was then computed on a pixel by pixel basis, neglecting pixels which failed to reach a threshold value. Ratios were computed every 10 seconds, or more frequently as needed. Average ratio values for each ROI (or cell) were written to disk for later analysis and graphing.

Calibration and computation of free Ca^{2+}

In vivo calibration of the fura-2/Ca^{2+} signal requires that the cell cultures be made permeable to Ca^{2+} in order to characterize the Ca^{2+}- fura-2 binding in the intracellular environment. In our hands, ionomycin (10 μM, Calbiochem) was not an effective Ca^{2+} ionophore in this preparation. As a result, we used a simpler, but less rigorous, calibration procedure. The fluorescence intensity at each wavelength was measured with a calcium-free and a calcium-saturated aliquot of buffered 10 μM fura-2 (K^+ salt) in 120 mM KCl, 10 mM HEPES, pH 7.4. These values were used to compute three terms: R_{min}, R_{max}, and β. From a series of calibrations carried out during these experiments, the average values of the calibration constants are: R_{min}=0.17, R_{max} = 3.56, and β = 4.81. The ratio data were transformed into Ca^{2+} concentrations by the equation of Grynkiewicz et al. (1985) assuming a K_d of 250 nM. Since this transformation already relies on a K_d assumption, we did not make any further corrections, such as for cytoplasmic viscosity (Poenie, 1990). Given these remaining uncertainties about the transformation, the reported $[Ca^{2+}]_i$ data must be taken only as estimates of the true values.

Experimental protocols

Influence of ACh on CBF and $[Ca^{2+}]_i$

Baseline CBF and $[Ca^{2+}]_i$ measurements were obtained for 5-10 minutes from cells bathed in HBSS. To exclude the possibility that fluid changes directly influence CBF and $[Ca^{2+}]_i$, a control exchange with HBSS alone was done. Whereas most solution changes did not produce any transient, a small transient in both $[Ca^{2+}]_i$ and CBF was seen in <5% of the cells and was always <50% of the amplitude of the transient observed in response to 10 μM ACh (data not shown). These transients are likely due to the previously reported mechanical sensitivity of ciliated cells (Sanderson and Dirksen, 1989; Sanderson et al., 1990). To minimize the probability of a mechanical artifact, solution exchanges were carried out gently and typically required 10-30 seconds for a 5-10 ml wash, which was sufficient to fully exchange the working chamber volume of ~0.5 ml.

During the CBF measurement, exposure to 10 μM ACh solutions was restricted to 2 minutes, after which the preparation was rinsed with ~10 ml of HBSS. Approximately 15 minutes later, $[Ca^{2+}]_i$ of the same cell was measured in HBSS and then in HBSS with 10 μM ACh. Thus we did not measure these two signals simultaneously. The order of CBF and $[Ca^{2+}]_i$ measurements was varied from coverslip to coverslip with no apparent difference.

Changing extracellular calcium concentrations

The '0 Ca^{2+}-HBSS' solution was prepared using divalent-free HBSS buffered with 1 mM EGTA and the free Mg^{2+} concentration was restored to 0.9 mM according to Fabiato and Fabiato (1978). If we assume that this solution had a total contaminating $CaCl_2$ concentration from all sources of ~1 μM, then the free Ca^{2+} concentration is likely to be 70 nM. Some early experiments were carried out with divalent-free HBSS containing 10 mM EGTA (resulting in an estimated free Ca^{2+} concentration of 8 pM). As this caused some cells to shrink and detach from the coverslip, the concentration of EGTA was lowered to 1 mM. Regular HBSS contained 1.3 mM Ca^{2+}.

For these experiments, baseline CBF and $[Ca^{2+}]_i$ data were obtained for 5-10 minutes in regular HBSS. Then the bathing solution was exchanged with 0 Ca^{2+}-HBSS and $[Ca^{2+}]_i$ was measured until a new stable level was reached at which point CBF was recorded. The bathing fluid was then exchanged with HBSS containing 10 mM $CaCl_2$ and the fura-2 signal was monitored until it was stable, at which time CBF measurements were repeated. For additional $[Ca^{2+}]_i$ and CBF recordings, several exchanges of 0 Ca^{2+}, 200 μM Ca^{2+}, and 10 mM Ca^{2+} solutions were performed.

Thapsigargin experiments

To empty IP_3-sensitive internal Ca^{2+} stores, the cells were treated with 1 μM thapsigargin (Thastrup et al., 1990). Since thapsigargin is an irreversible inhibitor, the immediate effect of thapsigargin alone could only be determined for one of the signals and different cells had to be used for the CBF and $[Ca^{2+}]_i$ measurements. For these experiments, however, the two recordings were done on the same day with coverslips of cells isolated from the same sheep. In experiments examining the effect of ACh after thapsigargin treatment, the bath fluid was exchanged with 10 μM ACh in HBSS. Controls were run with the thapsigargin solvent DMSO and no effect was observed.

RESULTS

Single cilium recording of CBF

The time course of the intensity signal from a single pixel focused above the basal body of a single cilium (Fig. 1A) is periodic (Fig. 1B) although not symmetric. The frequency can be estimated from the magnitude spectrum of a fast Fourier transform of this intensity signal (Fig. 1C). The maximum value corresponded to the beating frequency calculated from the peak-to-peak intervals in the intensity signal itself. By plotting each FFT magnitude spectrum as a vertical column against time and mapping the magnitudes in the third dimension with a normalized color look-up-table (Fig. 1D,E), the time course of CBF changes can be visualized. With this procedure, the measured CBF spectra show peaks of varying width indicating the degree of dispersion of beating frequency during the sampling interval. Occasionally these spectrographs revealed additional frequency components beside the main frequency. In some cases these frequencies were twice or three times the main frequency (Fig. 1), on other occasions they were randomly distributed (see, for example, Fig. 3B). Such information would be lost by plotting only the peak frequency as a function of time.

The CBF in these cultured cells is stable for at least 30 minutes as shown in Fig. 1. In fact, the cultures are extremely hardy and CBF measurements can be made from a single coverslip for many hours. Cilia were beating at 7.1±0.3 Hz at room temperature, 21-23°C (mean ± s.e.m.; n=31 cilia on 31 cells from 26 different coverslips plated from 16 tracheas; range = 3.0-10.4 Hz).

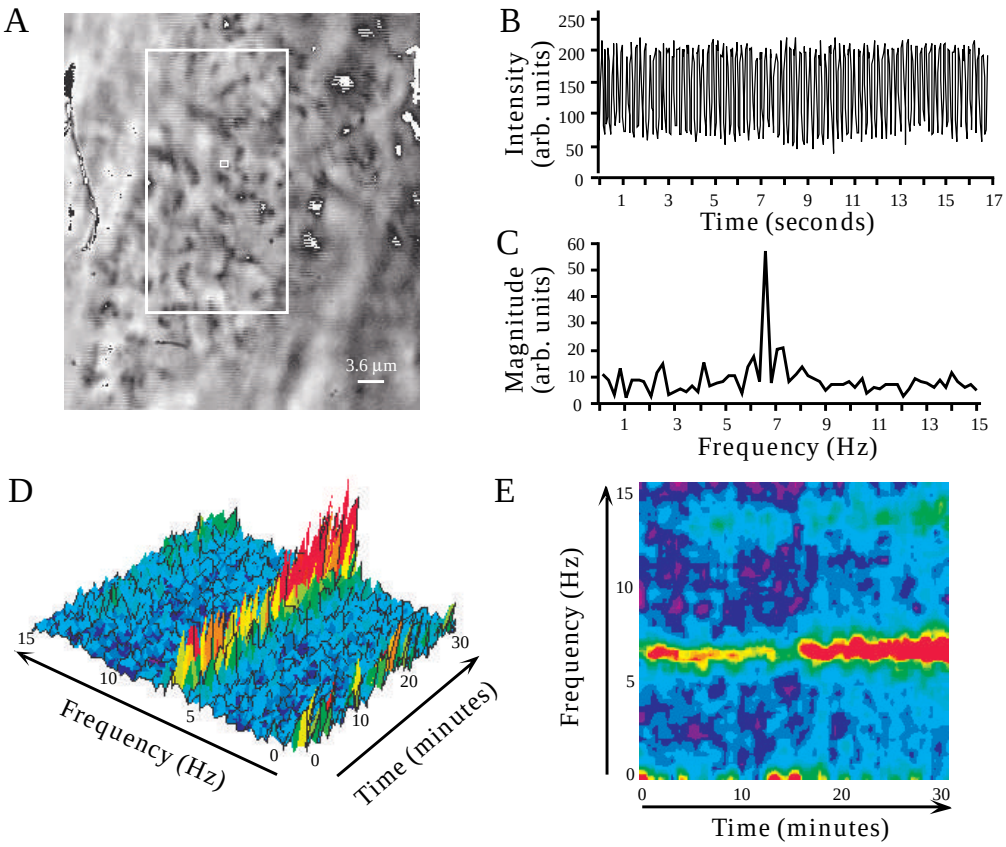

Fig. 1. Tracking changes in CBF with single pixel FFTs. (A) The digitized phase-contrast image of a ciliated cell is shown. A small box consisting of 9 pixels (indicated by a small white square) is placed right next to the basal body of a cilium in order to record CBF (see below and Materials and Methods). The larger square represents the area of the subsequent $[Ca^{2+}]_i$ measurement of the same cell using fura-2 ratio imaging. (B) The light intensity of one of the 9 pixels at the base of a cilium was recorded over time. (C) A single FFT magnitude spectrum calculated from 4.28 seconds of acquired intensity data. The peak of the spectrum shows that this cilium was beating at ~6.3 Hz. (D) When FFT magnitude spectra are collected repetitively, the time course of CBF can be constructed by plotting frequency vs time and using the FFT magnitude as the third dimension. This way of visualizing the data shows all the frequency components that influenced the intensity signal of that pixel. (E) The same plot as in (D) now viewed from the top, using color lookup tables as a measure for the magnitude value, normalized to show the maximum value in red and 0 in blue. The gap near the middle of the plot reflects a few minutes during which there was no clear peak in the FFT. The peak was recovered by adjusting the focus. This recording shows the stability of the beat frequency over >30 minutes.

Uniformity of CBF in a single cell

This CBF measurement method enabled us to select multiple independent pixels in order to simultaneously measure CBF of different cilia on the same cell. Repeated measurements in 5 different cells, looking at 3-8 single cilia per cell, showed that different cilia on the same cell beat at the same frequency (Fig. 2) within the frequency resolution of our measurement (i.e. 0.23 Hz, see Materials and Methods). In contrast, we have observed that different cells on the same coverslip, even ones only 50 µm apart, beat at frequencies as much as two- or three-fold different from one another (not shown).

Temperature-dependence of CBF

CBF is known to change in proportion to changes in temperature (Kennedy and Duckett, 1981; Eshel and Priel, 1986). In order to demonstrate that our method of single cilium recording can track changes in CBF, the temperature was adjusted from 12˚C to 33˚C using a Peltier device (model HCC-100A, Dagan Corp., Minneapolis, MN), and CBF was measured after the temperature in the bath was stabilized. Fig. 3A shows that at 22˚C this cilium was beating at 5.8±0.05 Hz (mean ± s.e.m. of maximum magnitude values, n=48 FFT spectra), while at 32˚C the CBF was 11.8±0.05 Hz (n=35). Therefore, the frequency approximately doubled every 10 deg. C. Beside the main frequency clearly seen in the figure, some harmonics also appear as mentioned above. The method of CBF measurement can also track the changes in CBF as shown by the response to warming and cooling in Fig. 3B. In this example, the frequency approached the bandwidth limit of our CBF tracking method when the temperature reached 28˚C.

Influence of ACh on $[Ca^{2+}]_i$ and CBF

To alter $[Ca^{2+}]_i$ and CBF via a receptor-coupled pathway, cultured cells were exposed to 10 µM ACh. Prior to addition of ACh, $[Ca^{2+}]_i$ was stable at a resting $[Ca^{2+}]_i$ of 53±9 nM (mean ± s.e.m., n=14 cells). ACh routinely increased $[Ca^{2+}]_i$ to a peak concentration of 146±12 nM or to 264±51% above baseline (Fig. 4). The time from the addition of 10 µM ACh to the peak increase in $[Ca^{2+}]_i$ was 0.5±0.1 minute. $[Ca^{2+}]_i$ relaxed back during ACh exposure with a time constant of 2.3±0.5 minutes (estimated by fitting only the data recorded in the presence of ACh). In 10 of 14 cells, the signal relaxed towards a higher plateau compared to the original baseline and only returned to the initial baseline after ACh was washed away. In the other 4 cells, the original baseline was reached while still in the presence of ACh.

CBF of the same cells showed a rise and relaxation similar to the $[Ca^{2+}]_i$ measurements in response to ACh. CBF increased from a baseline of 7±0.5 Hz to a maximum value of 9±0.2 Hz or to 31±2.8% above baseline as shown in Fig. 4. The time from the addition of 10 µM ACh to the peak increase in CBF was 0.3±0.05 minute. Given the time resolution of our measurements, this time-to-peak value cannot be considered different from the time to the peak of the $[Ca^{2+}]_i$ transient. CBF relaxed back towards the baseline in the continued presence of ACh with a time constant of 2.2±0.3 minutes, which is not

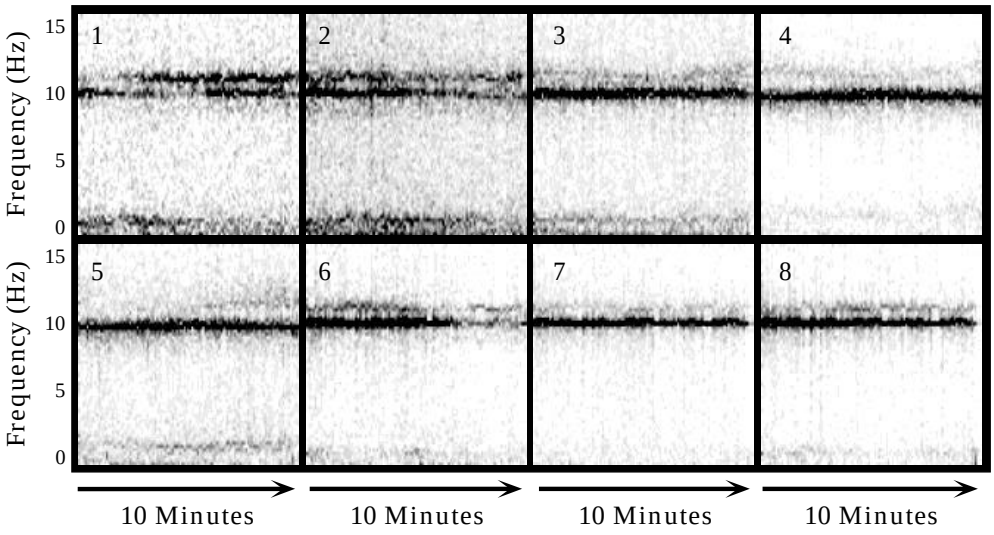

Fig. 2. Different cilia on the same cell beat with the same frequency. FFT magnitude plots of 8 different cilia tracked simultaneously on the same cell. CBF remains stable for all 8 cilia over an observation period of 10 minutes. With the exception of cilia 1 and 2, all cilia beat at the same frequency ±0.2 Hz. Cilia 1 and 2 also beat occasionally at an additional distinct frequency; each FFT spectrum revealed 2 distinct peaks as if the cilium hopped back and forth from one frequency to the other during the 4.28 second acquisition time of each spectrum.

different from the time constant of the $[Ca^{2+}]_i$ transient. In 10 of 14 cells, the initial baseline values were reached only after ACh was washed away; 9 of these 10 cells were the same ones that showed a higher $[Ca^{2+}]_i$ plateau, whereas 1 of the 10 cells did not show an elevated $[Ca^{2+}]_i$ plateau. In the other 4 cells, CBF values returned to the original baseline while ACh was still present, again closely resembling the $[Ca^{2+}]_i$ transients.

It is worth mentioning that a few cells showed a different pattern of response to ACh: both CBF and $[Ca^{2+}]_i$ levels went below baseline after brief and small initial transient rises. These results will be discussed elsewhere (M. Salathe, P. Ivonnet and R. J. Bookman, unpublished data).

These experiments show that both $[Ca^{2+}]_i$ and CBF respond to ACh in a similar fashion. Since the two measurements on each cell were made ~15 minutes apart, a direct individual comparison of these responses is not yet possible. Nonetheless, the basic *kinetic* features of the responses are the same: a rapid initial rise, a relaxation toward baseline, an elevated plateau level, and an eventual return to the original level. This establishes that muscarinic receptor activation by application of ACh produces kinetically similar changes in $[Ca^{2+}]_i$ and ciliary beating at the single cell level.

Influence of extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$)

To alter $[Ca^{2+}]_i$ in ciliated cells without G-protein activation and to check for associated CBF changes, the Ca^{2+} concentration in the extracellular fluid was varied. Upon initial exposure to the 0 Ca^{2+} (or 10 mM Ca^{2+}) solution, the fura-2 signal tended to decrease (or increase) slowly over the course of 10-20 minutes (not shown). Responses to subsequent changes in extracellular Ca^{2+} were typically quite rapid, regardless of whether the change added or removed Ca^{2+} (see Fig. 5B). In 0 Ca^{2+}, CBF and $[Ca^{2+}]_i$ of the same ciliated cells decreased compared to the levels in 10 mM Ca^{2+} (Fig. 5A,B). In this particular example, CBF was 3 Hz in 0 Ca^{2+} and jumped up to 6 Hz after the change to 10 mM extracellular Ca^{2+}. $[Ca^{2+}]_i$ in this cell increased from 42 to 97 nM with the solution exchange. The summary of the combined data in 0 Ca^{2+}, 200 μM Ca^{2+}, 1.3 mM Ca^{2+} (HBSS), and 10 mM Ca^{2+} is given in Table 1.

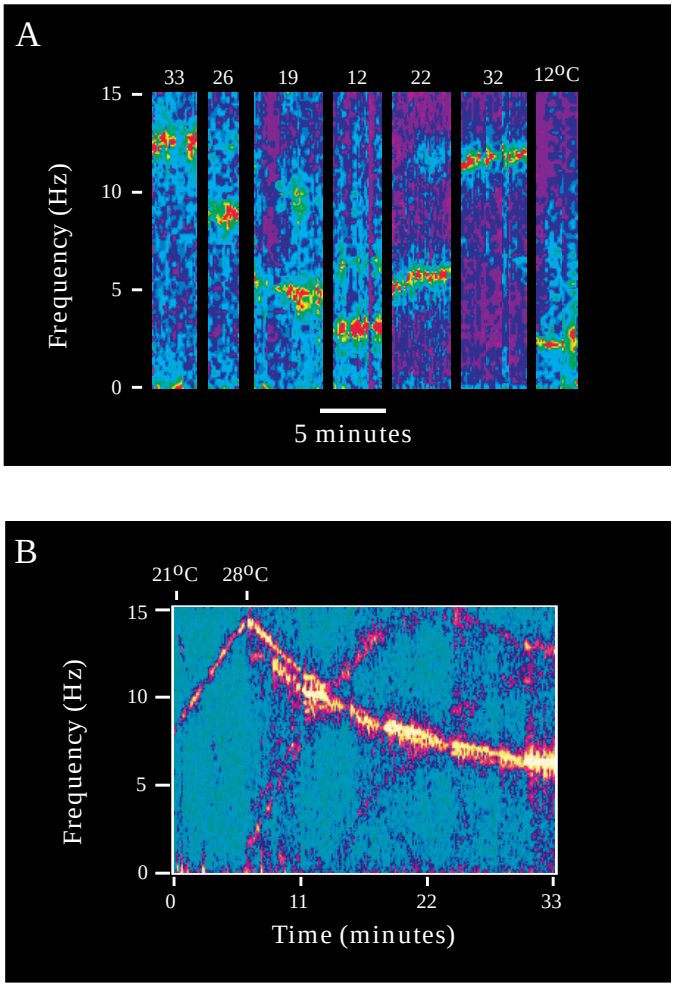

Fig. 3. Temperature dependence of CBF. CBF was measured after equilibration of the temperature at different levels as indicated in (A). At 22˚C, this cilium was beating at 5.8±0.05 Hz; at 32˚C at 11.8±0.05 Hz, illustrating that the frequency approximately doubles every 10 deg. C. In the 19˚C and left-hand 12˚C panel, harmonics at 2*f* are seen. (B**)** CBF changes can also be tracked during a temperature change. Again, some harmonics are seen in addition to the main frequency.

Table 1. Effect of extracellular Ca^{2+} on CBF and $[Ca^{2+}]_i$

$[Ca^{2+}]_o$	CBF (Hz)	$[Ca^{2+}]_i$ (nM)	Cells/coverslips
0	4.8±0.2	24±5	16/5
200 μM	5.9±0.5	30±3	6/2
1.3 mM	6.4±0.3	52±5	9/3
10.0 mM	6.9±0.3	87±11	10/5

Pooled data showing the effect of different extracellular Ca^{2+} solutions on CBF and $[Ca^{2+}]_i$. For each cell, CBF was measured from a single cilium. Values are given as mean ± s.e.m.; 20-22˚C.

Influence of thapsigargin on $[Ca^{2+}]_i$ and CBF

To alter $[Ca^{2+}]_i$ by a third method, the cells were exposed to thapsigargin, an irreversible blocker of the ER Ca^{2+}-ATPase (Thastrup et al., 1990). Exposure to thapsigargin caused an increase of $[Ca^{2+}]_i$ from a baseline of 76±5 nM to a peak value of 158±8 nM in 37 of 42 cells from 9 coverslips. The remaining five cells did not show a $[Ca^{2+}]_i$ response to thapsigargin. The time to the maximal $[Ca^{2+}]_i$ was 1.2±0.1 minutes. The $[Ca^{2+}]_i$ rise was transient and relaxed back towards baseline with a time constant of 1.8±0.2 minutes. In 17 cells (46%), a plateau higher than the original baseline was reached (Fig. 6A,B,C), whereas in 20 cells (54%) $[Ca^{2+}]_i$ levels returned to the original baseline. Addition of 1 μM thapsigargin also increased CBF in 8 of 9 cells from a baseline of 6.7±0.5 Hz to 9.1±0.5 Hz. The time to the maximal CBF was 0.8±0.1 minutes. The response was also transient, despite the maintained presence of thapsigargin, and relaxed back towards baseline with a time constant of 1.0±0.2 minutes. In 3 of the 8 cells (37%), a higher CBF plateau than the original baseline was maintained (Fig. 6A,B,C). In the other 5 cells, however, CBF reached the original baseline again. Thus, both CBF and $[Ca^{2+}]_i$ responses to thapsigargin had similar time courses, as indicated by the time to peak and the time constants of the relaxation phases (Fig. 6).

Effect of ACh on $[Ca^{2+}]_i$ and CBF after thapsigargin treatment

In order to determine whether the ciliostimulatory effect of ACh requires release of Ca^{2+} from internal stores, cells pretreated with thapsigargin to deplete the stores were exposed to 10 μM ACh. Such ACh addition resulted in a rapid *decrease* in $[Ca^{2+}]_i$ from a baseline of 79±5 nM (n=42 cells) to a minimum of 44±4 nM, followed by a slow return towards baseline (Fig. 7B). This decrease in $[Ca^{2+}]_i$ was observed in cells that showed elevated plateau responses to thapsigargin, in cells whose $[Ca^{2+}]_i$ levels returned to baseline prior to ACh addition, and in cells that did not show any $[Ca^{2+}]_i$ transient in response to thapsigargin. Interestingly, CBF also *decreased* upon addition of 10 μM ACh, from a resting level of 7.0±0.6 Hz (n=8 cells) to a minimum of 5.7± 0.1 Hz (Fig. 7A). Although the decrease in CBF upon ACh addition was modest, the effect was reproducible upon a second addition of ACh (not shown).

Oscillations

Early in culture (days 1-3 after plating), a majority of cells showed an unusual response to 10 μM ACh, namely that ACh produced $[Ca^{2+}]_i$ oscillations. In some coverslips, over 80% of the ciliated cells showed oscillatory fura-2 responses to ACh application. The same cells also showed oscillation (or more properly, frequency modulation) of the CBF response (Fig. 8). It is important to recall that these two measurements were not made simultaneously. However, both modulations showed the same average peak-to-peak interval (0.31 minute for the cell shown in Fig. 8). In other words, on a second-to-second basis, the levels of Ca^{2+} and CBF behave similarly in response to ACh.

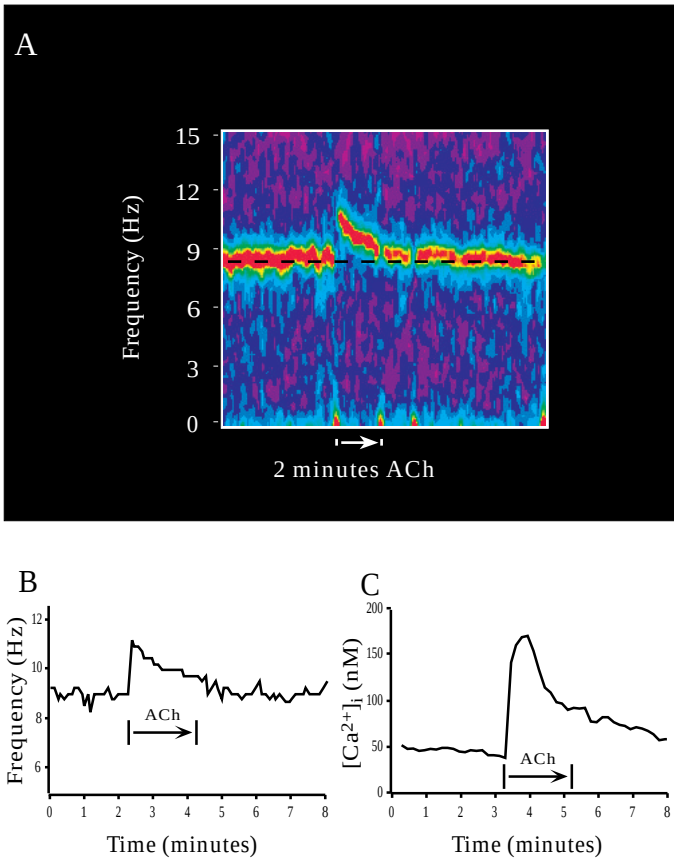

Fig. 4. Recording Ca^{2+} and CBF in response to ACh. CBF and fura-2 fluorescence recorded from a single cell. (A) The CBF response to the application of 10 μM ACh. The CBF spectra showed an 'immediate' rise, which then relaxed back towards the baseline, staying on a higher plateau in the presence of ACh. After 2 minutes in ACh, the coverslip was washed with ~10 ml of HBSS. (B) A plot of the frequency of the maximum of each spectrum from the complete FFT recording. (C) The $[Ca^{2+}]_i$ response of the same cell to the addition of 10 μM ACh ~15 minutes after the CBF measurement. The Ca^{2+} signal revealed a rise and relaxation towards baseline, similar to the CBF time course.

DISCUSSION

Single cilium recording of CBF

The analysis of intensity changes of transmitted light to estimate CBF has been widely used in experiments on ciliated cells (Dalhamn and Rylander, 1962; Yager et al., 1978; Kennedy and Duckett, 1981; Sanderson and Dirksen, 1985; Girard and Kennedy, 1986; Ben-Shimol et al., 1991). Most methods, however, measured light changes of several (about 10) ciliated cells. With the method of CBF measurement described here, the beating frequency of a single cilium can be

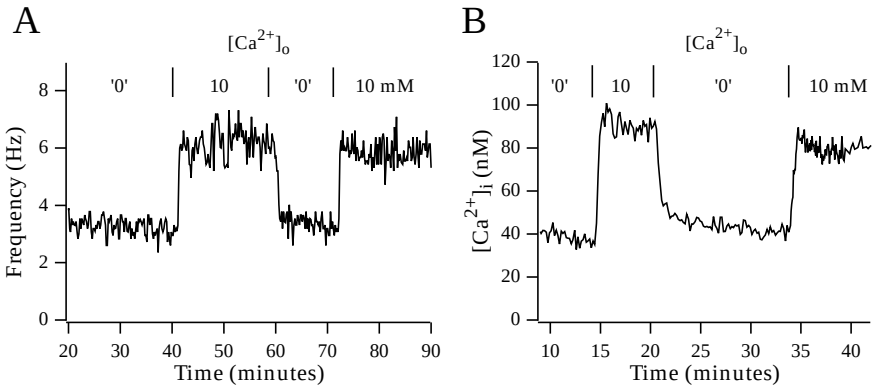

Fig. 5. Effect of extracellular Ca^{2+} on $[Ca^{2+}]_i$ and CBF. This figure illustrates the change of CBF (A; peak extracted plot) and $[Ca^{2+}]_i$ (B) secondary to exchanging the external fluid between '0 Ca^{2+}' and 10 mM Ca^{2+} solutions. The changes of $[Ca^{2+}]_i$ and CBF are quite abrupt. In this example, CBF doubled from 3 to 6 Hz. $[Ca^{2+}]_i$ of the same cell, measured ~3 minutes later, more than doubled (here from 42 nM to 97 nM). These changes are reproducible in the same cell several times.

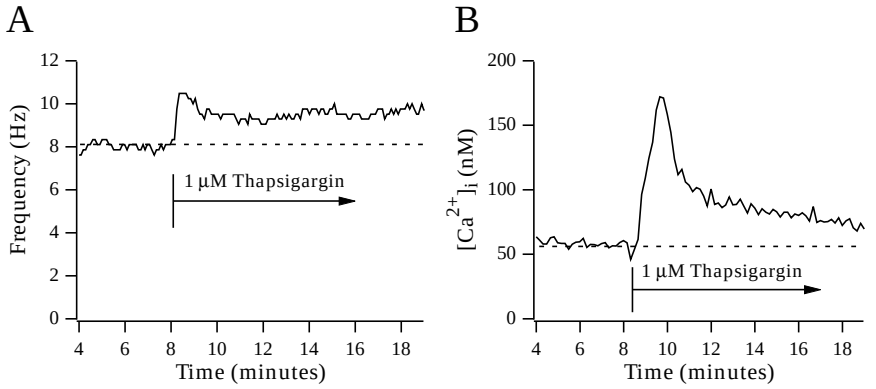

Fig. 6. Elevation and plateau of $[Ca^{2+}]_i$ and CBF with thapsigargin. At the time indicated, 1 μM thapsigargin was added to the HBSS solution. (A) The peak-extracted CBF plot and (B) the $[Ca^{2+}]_i$ plot. In this example, thapsigargin induced a transient in both CBF and $[Ca^{2+}]_i$, after which both signals returned to a plateau above the original baseline. Note that CBF and $[Ca^{2+}]_i$ measurements were not done in the same cell, since the transient only occurs once upon the addition of thapsigargin.

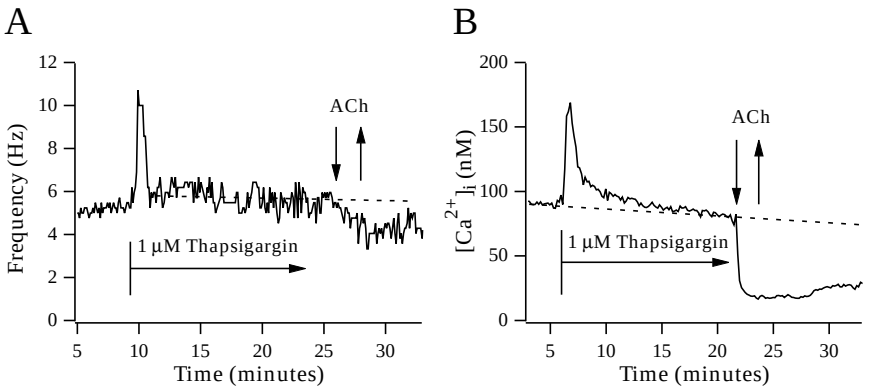

Fig. 7. $[Ca^{2+}]_i$ and CBF responses to ACh after treatment with thapsigargin. After an initial application of 1 μM thapsigargin to deplete the internal Ca^{2+} stores, the cells were exposed to 10 μM ACh. (A) The initial elevation of CBF (peak-extracted plot) in response to thapsigargin followed by a decrease in CBF upon addition of 10 μM ACh. The broken black line indicates the post-thapsigargin baseline. (B) The ACh-triggered drop in $[Ca^{2+}]_i$ from a different cell after treatment with thapsigargin.

recorded. The hardware capability that makes this possible is a combination of video digitizer and frame buffer that stores only the pixels selected by the user. With our system, we can track a large number of single pixels (or cilia) in this way, limited only by the time to compute the individual FFTs and store the data onto disk. Knowing that different cells of the same coverslip beat at different frequencies, large-area measurements may be insufficiently accurate to explore the molecular mechanisms that regulate CBF. Some investigators overcame this problem by tracking small areas covering only part of a ciliated cell and thus measuring a limited number of cilia (Sanderson and Dirksen, 1985; Ben-Shimol et al., 1991). Romet et al. (1991) reported a video-based method with a resolution similar to our system. With their method, taped video frames of a total 200× magnification were digitized and areas of 4 pixels analyzed for light intensity changes. This time-consuming off-line method, however, is inconvenient for long term recordings of CBF. Our ability to track the beating of a single cilium on-line for >60 minutes facilitates the correlation of ciliary activity with other single-cell measures such as $[Ca^{2+}]_i$ during experimental manipulations and solution changes.

One disadvantage is that our present instrument cannot measure $[Ca^{2+}]_i$ and CBF simultaneously, although we are working on an improved version to do so. Korngreen and Priel (1994) recently reported the results of their effort to measure CBF and $[Ca^{2+}]_i$ simultaneously, but the Ca^{2+} measurements were typically made from 3-4 cells. These improved instruments will allow a more detailed kinetic analysis of both signals in response to different stimuli.

An additional concern with our method is that a selected cilium may not be representative. Two arguments suggest that

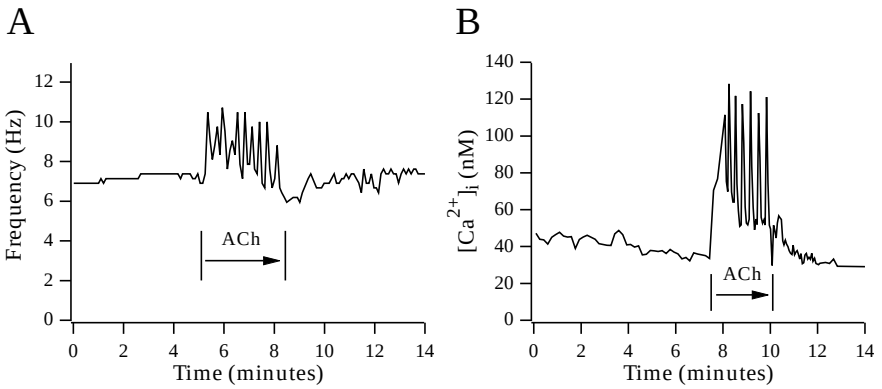

Fig. 8. Oscillations of Ca^{2+} and frequency modulation of ciliary beating. This figure illustrates the type of response that we have measured in 6 cells after 1-3 days in culture. Application of ACh produced not only an oscillation in the level of intracellular Ca^{2+} (B), but also a modulation of the CBF response (A). Although the records were not taken simultaneously, both signals showed an average peak-to-peak interval of 0.31 minute.

this is not the case. First, the experimenter, in selecting the cilium, can easily see on the video monitor whether the cilium seems grossly different from the others on the same cell. Secondly, we have recorded simultaneously from as many as 8 single cilia on the same cell and found that CBF, within measurement error, was the same for all (see Fig. 2).

Some features in the measured CBF magnitude spectra deserve to be mentioned. We have observed that in addition to the main beating frequency, *f*, there is often a clear signal at 2*f* and sometimes at 3*f*. The most likely cause of a 2*f* component is the pattern of movement at the base of the cilium, where we usually focus, which can intersect with the sampled pixel area twice per ciliary beat cycle rather than only once. The relative power at *f* and 2*f* reflects the degree of symmetry of the forward and recovery movements relative to the sampled pixel. An odd harmonic at 3*f* is likely to result from the sharp contrast between the basal body and the cytoplasm. This produces a rapid step change in the intensity signal. The Fourier transform of a step function contains significant contributions from the odd harmonics (Papoulis, 1977).

Regulation of CBF by $[Ca^{2+}]_i$

Although ACh has long been known to have a stimulatory effect on ciliary beating in tracheal cells (Corrsen and Allen, 1959), the results presented here are the first to demonstrate such stimulation in a mucus-free culture system. The results from these cultures therefore rule out the possibility that the CBF increase was secondary to a stimulation of mucus secretion. Addition of 10 μM ACh to the extracellular fluid produced *kinetically* similar transient increases in $[Ca^{2+}]_i$ and CBF, establishing a clear correlation at the single cell level. Such observations, however, do not reveal whether the changes in $[Ca^{2+}]_i$ and CBF are subsequent steps in the same signal transduction pathway and therefore causally related, or whether both changes are epiphenomena resulting from activation of two different pathways. Previous work suggested a correlation between $[Ca^{2+}]_i$ and CBF (Verdugo, 1980; Girard and Kennedy, 1986; Villalon et al., 1989; Di Benedetto et al., 1991; Lansley et al., 1992), but, as mentioned above, none of these studies provided data from single cells that could establish causal relationships. Several of our results suggest that it is the change in $[Ca^{2+}]_i$ that produces the change in CBF.

First, the close resemblance of the kinetics of the $[Ca^{2+}]_i$ and CBF responses to ACh, including the time to peak, the relaxation and the elevated baselines, supports the close coupling of $[Ca^{2+}]_i$ and CBF. The kinetic match between the signals

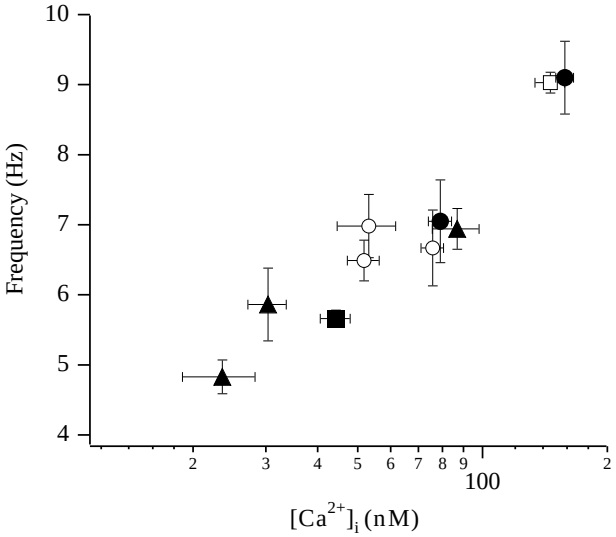

Fig. 9. Correlation between $[Ca^{2+}]_i$ and CBF. This figure summarizes the CBF and Ca^{2+} data. Plotted are the means ± s.e.m. of the CBF data versus the $[Ca^{2+}]_i$ data from all experiments. Open circles, HBSS measurements; open square, peak ACh response; filled circles, peak thapsigargin response and thapsigargin plateau; filled square, the ACh induced drop after thapsigargin treatment; triangles, measurements in different external Ca^{2+} concentrations ('0', 200 μM, 10 mM).

(although measured 15 minutes apart) is excellent. As mentioned above, a more detailed kinetic analysis, however, must await simultaneous $[Ca^{2+}]_i$ and CBF measurement. A lingering concern was that some other intracellular messenger (e.g. IP_3) might mediate the changes in CBF, since ACh was used to activate a G-protein-coupled muscarinic receptor. In fact, many substances reported to increase both $[Ca^{2+}]_i$ and CBF (e.g. ATP, ACh, adrenergic drugs, mechanical stimulation) increase IP_3; therefore the change in $[Ca^{2+}]_i$ might be nothing more than an epiphenomenon. Other evidence presented here, however, argues strongly against this.

For example, we demonstrated that subsequent to depletion of intracellular Ca^{2+} stores by thapsigargin, addition of ACh to the bathing fluid produced *decreases* in both $[Ca^{2+}]_i$ and CBF. If ACh changed CBF independently of $[Ca^{2+}]_i$, an increase in CBF would be expected. Thus, this drop in both $[Ca^{2+}]_i$ and CBF is probably the strongest evidence to date that it is the elevation of $[Ca^{2+}]_i$ that is responsible for the 'normal' stimulatory effect of ACh on CBF. Although the mechanism respon-

sible for the ACh-stimulated decrease in $[Ca^{2+}]_i$ remains a subject for future investigation, the effect is clear and has not been described previously. In the physiological context, this suggests that the action of ACh on CBF should not be viewed as purely stimulatory. Rather, cholinergic agonists can exert more subtle forms of control and the effect of ACh on CBF will depend, in part, on the state of repletion of the internal Ca^{2+} stores.

Then, we showed that elevation of $[Ca^{2+}]_i$ by two other methods, independent of G-protein activation (thapsigargin and increasing $[Ca^{2+}]_o$), caused associated increases in CBF. These two methods rely on different sources of Ca^{2+} and produced changes in CBF proportional to the $[Ca^{2+}]_i$ changes. Therefore, G-protein activation is not required for CBF increases.

Finally, our observation of ACh-triggered Ca^{2+} oscillations and matching frequency modulation in the CBF signal shows both an interesting physiological consequence of Ca^{2+} oscillations and a further example that CBF can follow even fairly rapid $[Ca^{2+}]_i$ changes.

In summary, every manipulation that increased $[Ca^{2+}]_i$ caused an increase in CBF and every manipulation that produced decreases in $[Ca^{2+}]_i$ produced decreases in CBF. Fig. 9 shows a summary of the data from all experiments and demonstrates the strong link between the two signals. Taken together, these results support the hypothesis that Ca^{2+} is an essential messenger in the regulation of CBF.

It remains unclear, at the molecular level, how changes in $[Ca^{2+}]_i$ are transduced into changes in CBF and the answer cannot be directly deduced from Fig. 9. Some reports have provided evidence, using calmodulin inhibitors, that $[Ca^{2+}]_i$ acts through calmodulin (Verdugo, 1980; Di Benedetto et al., 1991) and possibly activates Ca^{2+}/calmodulin-dependent protein kinase with subsequent phosphorylation of a ciliary target (Hirano-Ohnishi and Watanabe, 1989). An alternative explanation is that a calcium-binding protein (perhaps even calmodulin) mediates the Ca^{2+} action directly or through a pathway independent of Ca^{2+}/calmodulin-dependent kinase. For example, it is possible that nitric oxide (NO) might mediate the $[Ca^{2+}]_i$-induced CBF change, since: (a) calmodulin can stimulate nitric oxide synthase (NOS; Bredt and Snyder, 1990); (b) increased NO production has been associated with an increase in CBF (Jain et al., 1993); and (c) ciliated cells, including those used in the present experiments, stain for NADPH-diaphorase activity, suggesting that they contain NOS (Kobzik et al., 1993; M. Salathe, ?. Michael, ?. Marino and R. J. Bookman, unpublished observations). Thus while it seems clear that $[Ca^{2+}]_i$ plays a central role in regulating CBF in ciliated tracheal cells, the Ca^{2+} sensor and the molecular effectors for modulating CBF remain to be identified.

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