Regulation of Airway Ciliary Activity by Ca^{2+}: Simultaneous Measurement of Beat Frequency and Intracellular Ca^{2+}

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ABSTRACT Airway ciliary activity is influenced by $[Ca^{2+}]_i$, but this mechanism is not fully understood. To investigate this relationship, ciliary activity and $[Ca^{2+}]_i$ were measured simultaneously from airway epithelial ciliated cells. Ciliary beat frequency was determined, for each beat cycle, with phase-contrast optics and high-speed video imaging (at 240 images s^{-1}) and correlated with $[Ca^{2+}]_i$ determined, at the ciliary base, by fast imaging (30 images s^{-1}) of fura-2 fluorescence. As a mechanically induced intercellular Ca^{2+} wave propagated through adjacent cells, $[Ca^{2+}]_i$ was elevated from a baseline concentration of 45 to 100 nM, to a peak level of up to 650 nM. When the Ca^{2+} wave reached the ciliary base, the beat frequency rapidly increased, within a few beat cycles, from a basal rate of 6.4 to 11.6 Hz at 20–23°C, and from 17.2 to 26.7 Hz at 37°C. Changes in $[Ca^{2+}]_i$, above 350 nM, had no effect on the maximum beat frequency. We suggest that airway ciliary beat frequency is 1) controlled by a low range of $[Ca^{2+}]_i$ acting directly at an axonemal site at the ciliary base and 2) that a maximum frequency is induced by a change in $[Ca^{2+}]_i$ of ~250–300 nM.

INTRODUCTION

The function of airway cilia is the expulsion of inhaled contaminants (Wanner et al., 1996; Sanderson, 1997). This is achieved by the propulsion of a mucus layer, with entrapped particles, toward the esophagus by the repetitive "clawing" action of the underlying cilia. The efficiency and speed of mucus clearance are dependent on a cooperative metachronal organization of the ciliary activity and the rate of the ciliary beat frequency. Although the regulation of ciliary beat frequency is important for the maintenance of lung health, and a wide variety of drugs or stimuli are known to change airway ciliary activity, little is understood regarding the intracellular mechanisms controlling beat frequency. While it is important to catalogue the pharmacological and physiological responses of airway ciliated cells to these drugs and stimuli, it is the intracellular second messengers produced in response to the drugs and stimuli, for example Ca^{2+}, IP_3, cAMP, or NO, that dictate the cellular mechanism of action and are the key to understanding how axonemal modifications induce changes in beat frequency.

A change in $[Ca^{2+}]_i$ appears to be a fundamental mechanism for altering ciliary activity (Tamm, 1994). For example, in protozoa, such as *Paramecium* (Nakaoka et al., 1984; Bonini et al., 1991; Hinrichsen, 1993) and *Chlamydomonas* (Wakabayashi et al., 1997; Witman, 1993), and ctenophores (Tamm and Terasaki, 1994; Tamm, 1994), elevated Ca^{2+} induces an increase in beat frequency and a reversal or change in beat pattern. In contrast, in the gill cilia of mussels, elevated Ca^{2+} induces ciliary arrest (Walter and Satir, 1978). In mammalian airway ciliated epithelial cells, many studies clearly indicate that ciliary beat frequency is increased by elevations in $[Ca^{2+}]_i$, although the direction of the ciliary beating is unaffected (Lansley et al., 1992; Korngreen and Priel, 1994, 1996; Sanderson and Dirksen, 1989; Salathe et al., 1997; Salathe and Bookman, 1995; Girard and Kennedy, 1986; Kakuta et al., 1985; Di Benedetto et al., 1991). Oviduct cilia appear to have a similar Ca^{2+} sensitivity (Verdugo, 1980; Villalon et al., 1989). The extracellular stimuli that initiate elevations in $[Ca^{2+}]_i$ in mammalian cells include extracellular ATP (Villalon et al., 1989; Korngreen and Priel, 1996; Korngreen et al., 1998; Evans and Sanderson, 1999), muscarinic agonists (Salathe et al., 1997; Salathe and Bookman, 1995), bradykinin (Paradiso et al., 1991), and mechanical stimulation (Sanderson et al., 1990).

The relationship between changes in $[Ca^{2+}]_i$ and ciliary beat frequency has remained difficult to define because of the difficulties of simultaneously measuring $[Ca^{2+}]_i$ and ciliary beat frequency from the same location. Recordings of ciliary activity are generally made by monitoring the fluctuations in light intensity of the microscopic image (Sanderson and Dirksen, 1985); video microscopy appears to be ideal for this purpose. However, because cilia move at rates of up to 30 Hz, the frame rate of normal video (30 fps) is generally too slow, according to the Nyquist regime, to provide the sampling rate required to accurately reproduce the ciliary signal (Inoue and Spring, 1997). Similar considerations must be taken into account when changes in $[Ca^{2+}]_i$ are recorded, as these changes often occur as intracellular or intercellular waves that sweep across cells at velocities on the order of 20 $\mu m\ s^{-1}$ (Thomas et al., 1996).

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Ratiometric measurements required to quantify $[Ca^{2+}]_i$ are difficult to perform at video rates.

Although a number of groups (Villalon et al., 1989; Korngreen and Priel, 1994; Salathe and Bookman, 1995; Mao and Wong, 1998) have developed systems to measure $[Ca^{2+}]_i$ and ciliary beat frequency, these techniques have limited temporal or spatial resolution. For example, $[Ca^{2+}]_i$ measurements were averaged from areas encompassing one or more cells (Korngreen and Priel, 1994; Salathe and Bookman, 1995; Mao and Wong, 1998; Villalon et al., 1989), and only average $[Ca^{2+}]_i$ could be associated with changes in ciliary beat frequency. Similarly, ciliary beat frequency was determined with a low time resolution, requiring 1–2 s to compute from a fast Fourier transform. Thus rapid changes in ciliary beat frequency could not be detected.

To meet the high spatial and temporal requirements to measure $[Ca^{2+}]_i$ and ciliary beat frequency, we have developed a high-speed imaging technique that simultaneously records phase-contrast images at 240 images s^{-1} and fluorescence images at 30 images s^{-1}. High-speed phase-contrast images provide the necessary time resolution to measure the period of each ciliary beat cycle and its components (both at 25°C and 37°C), whereas the fluorescence images record the dynamic changes of $[Ca^{2+}]_i$ at specific locations within the cell. With this technique, we found that maximum changes in airway ciliary beat frequency were induced by relatively small, local changes in $[Ca^{2+}]_i$ and occurred within a few beat cycles. The speed of the change in beat frequency would indicate a direct action of Ca^{2+} on the axoneme, whereas the cellular location of the changing $[Ca^{2+}]_i$ suggests that the Ca^{2+} action may be initially at the ciliary base.

MATERIALS AND METHODS

All of the methods used have been described in detail elsewhere and are only briefly reviewed.

Tissue culture

Airway epithelial cells were obtained from the tracheas of New Zealand White rabbits. After removal of the trachea, the mucosal layer was dissected from the trachea, cut into small explants, and cultured on collagen-coated cover glasses. The preparation of the collagen-coated cover glasses was modified slightly from the original technique (Sanderson and Dirksen, 1985) by the substitution of formaldehyde for gluteraldehyde (5%) as the fixative used to stabilize the collagen gel. Outgrowths of ciliated cells were ready for use after ~5–10 days (Sanderson and Dirksen, 1985; Sanderson et al., 1990; Dirksen et al., 1995). Cells were loaded with fura-2 by incubation in Hanks' balanced salt solution, without phenol red, supplemented with 25 mM HEPES (sHBSS), containing 5 μM fura-2-AM for 1–2 h at 37°C. Cells were washed with sHBSS and allowed a minimum of 30 min to deesterify the fura-2-AM before experimentation (Sanderson et al., 1990).

High-speed phase-contrast video imaging

To correlate ciliary beat frequency with $[Ca^{2+}]_i$, high-speed phase-contrast images (240 images s^{-1}) of the cilia were obtained simultaneously with fast fluorescence images (30 images s^{-1}) of fura-2. Cells were observed on an inverted microscope (Nikon Diaphot) with a 40× 1.3 N.A, oil Ph 4 objective. Phase-contrast images were acquired with a charged-coupled device (CCD) camera (Sanyo, VDC3800) custom modified for high-speed imaging and recorded with an optical memory disc recorder (OMDR) (Panasonic TQ3031F), using red light generated by filtration of a halogen light source through a 645-nm long-pass filter (Fig. 1). The generation of 240 images s^{-1} with standard video was achieved by prematurely resetting the vertical synchronization pulse three times in each video field (1/60 s). This resulted in a video output that had eight images in each video frame (four per field, two fields per image) (Fig. 2). These interlaced, high-speed images were fully compatible with standard RS-170 video signals and, as

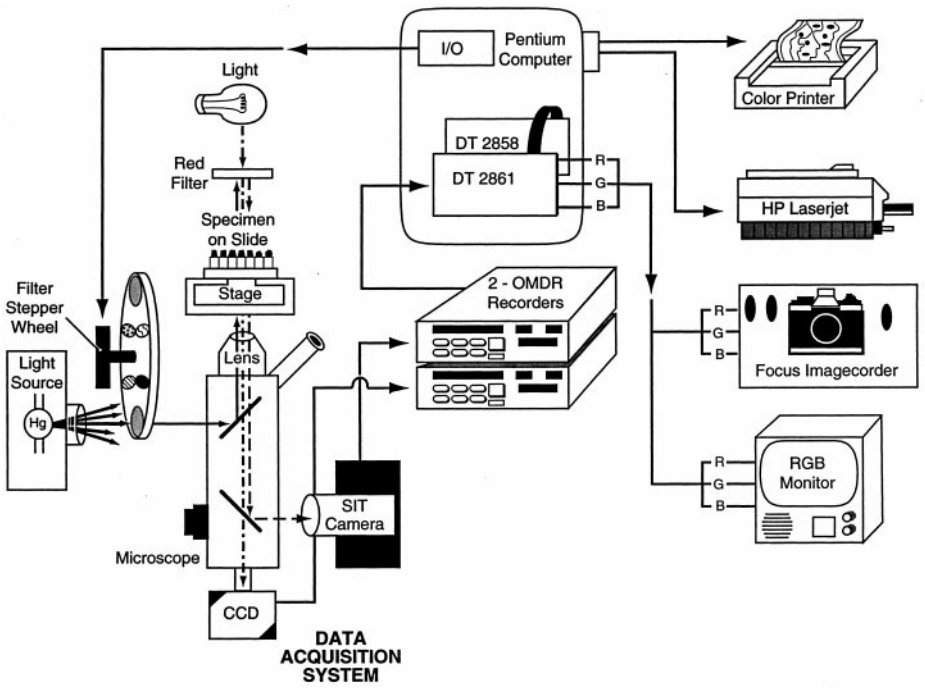

FIGURE 1 A general schematic of the system used to perform simultaneous high-speed phase-contrast imaging (240 images s^{-1}) with fast fura-2 fluorescence imaging (30 images s^{-1}). Red light (– · –) illuminates the specimen and passes through the microscope to the CCD camera. The excitation light (——) is reflected to the specimen by a 400-nm dichroic mirror. Emitted fluorescent light (510–520 nm, – – –) passes through the 400-nm dichroic mirror but is reflected to the SIT camera by a 655-nm dichroic mirror. Dual OMDRs record the video signal from each camera simultaneously. Data analysis is performed with a PC and frame grabbers (DT2861/2868). Images are viewed with peripheral devices.

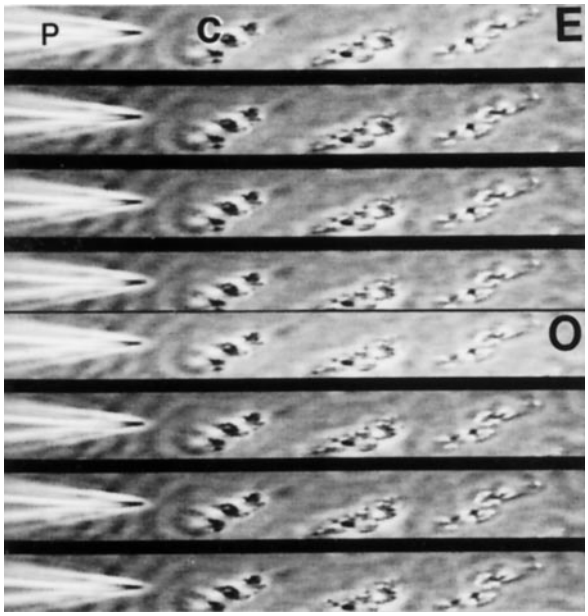

FIGURE 2 A series of eight sequential high-speed phase-contrast images after digital processing to separate the even (E) and odd (O) video fields originally saved as a single video frame. The micropipette or probe (P) is to the left, and three ciliated (C) cells are to the right. The positions of cell outlines and points for analysis are shown in Fig. 4. Each panel is acquired at an interval of 4.166 ms (1/240 s). Panel width = 83.5 μm; panel height = 26 μm. Horizontal and vertical scales differ because of the 4:3 aspect ratio of video images.

a result, could be recorded and analyzed with conventional video equipment. The ciliary activity was measured by digitizing the images and extracting the variation in gray-level intensity, with respect to time, of an area of 0.9 by 0.7 μm (3 $\times$ 3 pixels, magnification $\times$ 1645, horizontal scale: 155 μm = 512 pixels; vertical scale: 110 μm = 480 pixels) that coincided with a position ~⅓-½ of the way along the length of the cilium in its resting position. Because the recorded video fields were interlaced, it was necessary to separate individual video fields to reconstruct and view the actual temporal sequence of ciliary motion. Individual images were sequentially presented by the OMDR and digitized by a frame grabber (Data Translation, DT2861). The odd and even video lines of each video field were extracted, repositioned to close-up adjacent lines (lines 1, 3, 5, to 479 become 1, 2, 3, to 240, and lines 2, 4, 6. . . become 241, 242, 243, to 480), and relocated to another image frame (Fig. 2). Ciliary beat frequency was obtained from a gray-level analysis at selected pixel points (Figs. 3 and 4), but it was not necessary to reconstruct each image for this analysis, because the location of the pixel points repeated at a known location in each image and the gray level at these points could be determined in sequence. A major and unique characteristic of this analytical technique is that the ciliary beat frequency of multiple cells can be recorded simultaneously with high temporal resolution.

Fast fluorescence imaging

Fluorescence images were generated by the excitation of fura-2 trapped within cells with 340- or 380-nm light generated by filtration of a Hg arc lamp. The 510-nm fluorescence light emitted by fura-2 was selectively directed to a silicon intensified target (SIT) camera (Cohu, San Diego) with a 655-nm-cutoff dichroic mirror. The phase-contrast and fluorescence images were simultaneously recorded on separate OMDRs under the control of a synchronizing data acquisition program. This technique is described in detail elsewhere (Sanderson and Dirksen, 1995; Sanderson, 1998). The synchronization of the two recording OMDRs was examined by recording a sequence of phase-contrast images of the deflection of stimulating glass micropipette simultaneously on each OMDR. With this technique, it was found that the OMDR recording of ciliary activity lagged the OMDR recording of Ca^{2+} changes by an average of 111 ms (n = 3), and the temporal data were adjusted accordingly.

The correlation of changes in $[Ca^{2+}]_i$ with changes in the activity of specific small groups of cilia within one cell were determined by the careful alignment of the CCD and SIT camera images. A grid reticule or 1-μm-diameter fluorescence latex beads (Molecular Probes) were used to identify and align reference points in each image. The precise relationship between the sites of Ca^{2+} and ciliary beat frequency measurements was also confirmed by recording a sequence of phase-contrast images of the ciliary activity with the SIT camera.

The localized changes in $[Ca^{2+}]_i$ were determined at the base of the cilia from an area of 1.8 $\times$ 1.4 μm (6 $\times$ 6 pixels, magnification $\times$ 1645) and calculated from the change in fluorescence at a single wavelength, using a modified version of the formulae derived by Grynkiewicz et al. (1985). Images were corrected for background illumination, shading, and any dye loss due to photobleaching. The details of this technique are described by Leybaert et al. (1998). An in vitro calibration procedure was used to relate $[Ca^{2+}]_i$ to fluorescence ratios (Sanderson et al., 1990). A reproducible thin layer of dye solution was required for the in vitro calibration, and this was achieved by constructing a chamber from two cover glasses, using plastic wrap as a spacer. One side of one coverslip (40 $\times$ 22 mm, no. 1) was covered completely with wrap. A thin channel was scored in the wrap with a razor blade, and the intervening wrap was removed with forceps. A 15-mm circular coverslip was placed over the channel, and pressure was applied to the edges to form a seal with the wrap. The appropriate fura-2 solutions with zero and 1 mM Ca^{2+} were then drawn through the chamber, via capillary action, to obtain the respective ratios at 0 (R_{min}) and 1 mM Ca^{2+} (R_{max}).

Mechanical stimulation to increase $[Ca^{2+}]_i$ and ciliary beat frequency

Mechanical stimulation is a simple and reliable protocol for the induction of increases in $[Ca^{2+}]_i$ at specific locations in cells in tissue culture. A glass micropipette (tip diameter ~1–2 μm) was attached to a piezoelectric device and used to distort the apical membrane of a cell. The duration and displacement of the micropipette deflection were controlled by an electrical pulse applied for 100–150 ms to the piezoelectric device (Sanderson and Dirksen, 1986; Sanderson et al., 1990). The cells were stimulated at either room temperature (20–23°C) or 37°C.

Cultures were maintained at 37°C with a heating element attached to the objective. The thermal contact between the objective and the cover glass was made by immersion oil. Temperature was regulated and monitored with a temperature controller (Omega Engineering, Stamford, CT; CN7600 series) and a miniature T-thermocouple placed, with the aid of a micromanipulator, in the HBSS, just above the cells.

RESULTS

Mechanical stimulation to elevate $[Ca^{2+}]_i$ and ciliary beat frequency

In previous studies, it was demonstrated that a mechanical stimulus, applied to a single cell, initiated an increase in $[Ca^{2+}]_i$ that propagated to adjacent cells as an intercellular Ca^{2+} wave (Sanderson and Dirksen, 1986; Sanderson et al., 1988, 1990; Sanderson, 1997). This ability to precisely control the initiation of temporal and spatial changes in $[Ca^{2+}]_i$ was exploited in the design of our experiments to correlate changes in $[Ca^{2+}]_i$ with ciliary beat frequency. Multiple ciliated cells were aligned along the long axis of

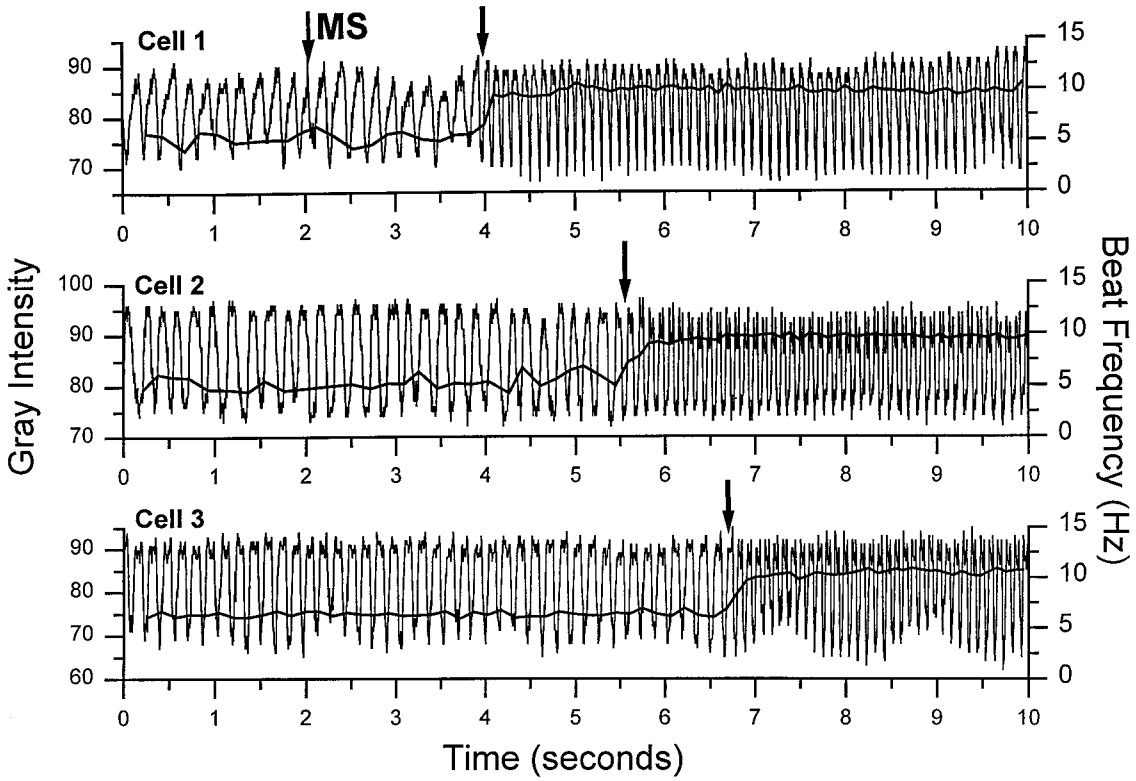

FIGURE 3 The digital wave form of gray intensity with respect to time, which represents ciliary activity. The three traces represent the activity of the three cells shown in Figs. 2 and 4. Cell 1 is closest to the micropipette; cell 3 is the furthest from the micropipette. After mechanical stimulation (*arrow*, MS), an intercellular Ca^{2+} wave propagates from cell 1 to cells 2 and 3 (Fig. 4). As the Ca^{2+} wave passes each analysis point, the ciliary activity is increased as indicated by the decrease in the period of the wave form (*arrow* on each trace). The Ca^{2+} wave takes ~½-1 s to propagate between cells, and this accounts for the sequential change in beat rate from cell 1 to cell 3. Each trace shows that the decrease in the wave form period is complete within a few beat cycles.

the field of view of the camera (across the top of the screen), and a single cell, at one end of the area, was mechanically stimulated (Figs. 2 and 4). The mechanical stimulus initiated a wave of increased $[Ca^{2+}]_i$ that spread across the field of view, which in turn induced an increase in ciliary activity as it propagated (Figs. 3 and 4). As a result, a single stimulus was used to initiate sequential changes in ciliary beat frequency and $[Ca^{2+}]_i$ in different cells in separate experiments.

Conventionally, the rate of ciliary activity is defined in units of frequency (Hertz), and this implies counting the number of beat cycles per second. However, our high-speed video recordings allow the measurement of the duration or period of each beat cycle. As a result, the beat frequencies reported in this study are calculated, every beat cycle, from the duration of the beat cycle (1/period). The importance and advantages of this approach, unlike averaging techniques such as those using fast Fourier transforms or long sampling periods, are that it reveals rapid changes in ciliary beat activity.

As a Ca^{2+} wave propagated through the cells, it elevated the $[Ca^{2+}]_i$ (Figs. 4–6), at room temperature (20–23°C), from a mean resting level of 68 nM (Table 1, range ~20–200 nM) to a mean peak level of 387 nm (Table 1, ranging from 300 to 800 nM). Similarly, the ciliary beat frequency increased from a mean base value of 6.4 Hz to a mean peak value of 11.6 Hz (Table 1) as the Ca^{2+} wave propagated through each ciliated cell (Figs. 4 and 5). At 37°C, the resting $[Ca^{2+}]_i$ was elevated to ~207 nM, and the mean peak $[Ca^{2+}]_i$ associated with a Ca^{2+} wave was 382 nM. The corresponding mean basal ciliary beat frequency was 17.2 Hz, and this was increased to 26 Hz by the Ca^{2+} wave (Table 1). Although the basal $[Ca^{2+}]_i$ was elevated by temperature, the mean maximum $[Ca^{2+}]_i$ associated with a Ca^{2+} wave appeared to be independent of temperature (Table 1). In contrast, ciliary beat frequency was elevated by increases in $[Ca^{2+}]_i$ and temperature. These results suggest that airway ciliary beat frequency is independently regulated by temperature and $[Ca^{2+}]_i$.

Threshold activation of increases in ciliary beat frequency

By determining the beat frequency or period for each beat cycle, we found that the actual change in ciliary beat rate occurred very rapidly. However, this rapid change in the ciliary beat frequency, in cells distal to the stimulated cell,

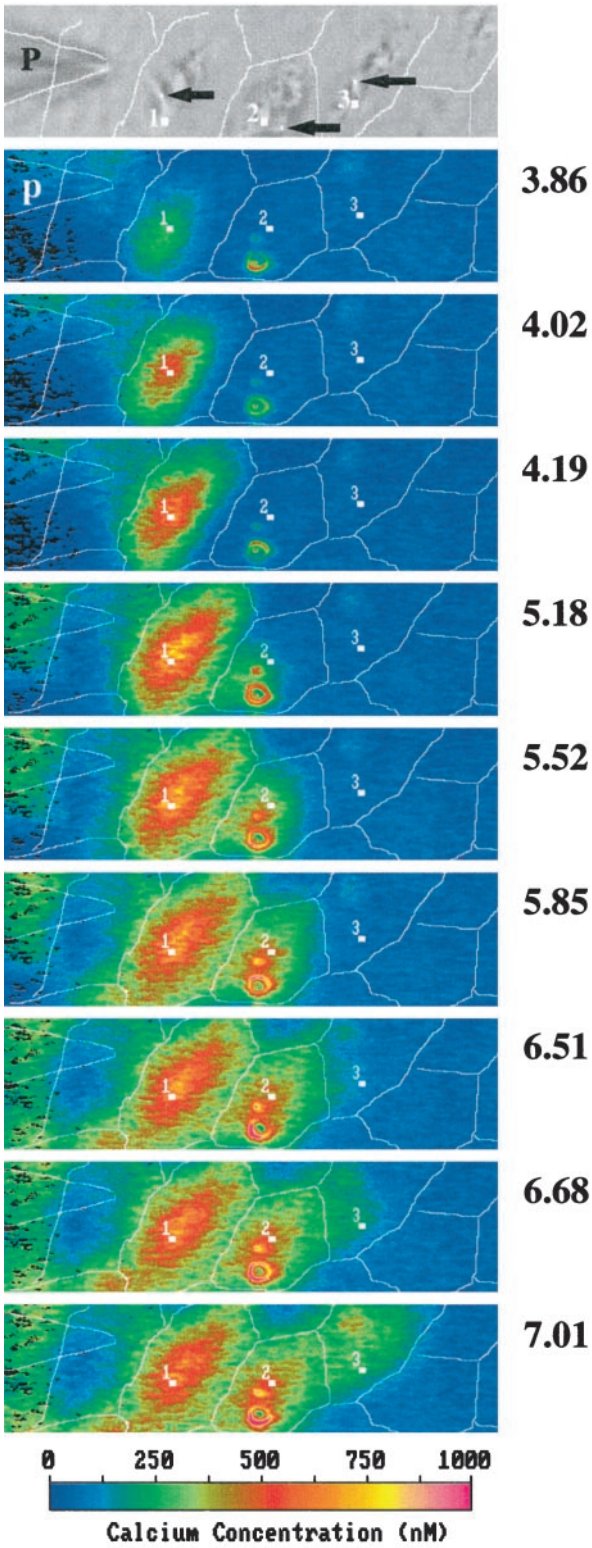

FIGURE 4 An intercellular Ca^{2+} wave initiated by mechanical stimulation sequentially elevates ciliary beat frequency. (*Top*) A phase-contrast image of the ciliated cells and the micropipette or probe (p) used for mechanical stimulation. Cell outlines are shown in white. The white dots indicated by the black arrows represent the position and detector size where the ciliary activity (Fig. 3) was analyzed. The $[Ca^{2+}]_i$ was analyzed at the base of the cilia, as indicated by the numbered white squares. Color images show the sequential changes in $[Ca^{2+}]_i$ (*top to bottom*) associated with the propagation of an intercellular Ca^{2+} wave from cell to cell. In this particular example, the $[Ca^{2+}]_i$ response of the stimulated cell was weak, but the response of the adjacent cells was strong. The time (in seconds), from the beginning of the experiment, at which each image was obtained is indicated at the side of each panel. Mechanical stimulation was applied 2.2 s after the initiation of recording (MS *arrow*; Fig. 3). A color calibration bar for $[Ca^{2+}]_i$ is indicated at the bottom. Panel width = 121 μm: panel height = 32.5 μm. Vertical and horizontal scales differ because of the 4:3 aspect ratio of video images.

occurred only after a time delay that was proportional to the distance of the cilia of interest from the stimulated cell (Figs. 4 and 5). This delay resulted primarily from the time taken for the intercellular Ca^{2+} wave to propagate through the cells (Sanderson et al., 1988, 1990). In all cases the beat frequency increased only after the $[Ca^{2+}]_i$ had increased (Figs. 4 and 5 at 20–23°C, Figs. 7 and 8 at 37°C). The increase in beat frequency was delayed by 0.265 ± 0.206 s (mean ± SD, n = 11) at 20–23°C and 0.092 ± 0.048 s (mean ± SD, n = 6) at 37°C after the $[Ca^{2+}]_i$ had begun to increase at the base of the cilium. Frequently, the increase in beat frequency occurred within one to three beat cycles at room temperature (Fig. 3). The beat period decreased from ~180 to ~100 ms from one beat cycle to the next (equivalent to a beat frequency increase of 5.55 to 10.0 Hz). The mean time taken for ciliary beat frequency to increase from the resting frequency to the maximum frequency was 0.355 ± 0.123 s (n = 11). At 37°C the beat frequency increased over three to six beat cycles (mean time = 0.160 ± 0.082 s) (Fig. 7), but it should be pointed out that each beat period at 37°C is shorter than at 20–23°C because of the temperature-induced increase in beat frequency.

Our results indicate that at room temperature the maximum increase in the beat frequency was induced once $[Ca^{2+}]_i$ exceeded ~300 nM (ranging from 150 to 400 nM) (Figs. 4–8). Larger increases in $[Ca^{2+}]_i$ did not increase the beat frequency further (Figs. 5 and 6). However, the larger elevations in $[Ca^{2+}]_i$ maintained elevated beat frequencies for extended periods. The response of cell 1 in Fig. 5 indicates that the $[Ca^{2+}]_i$ can fall substantially with only a small effect on beat rate. When the $[Ca^{2+}]_i$ is plotted against ciliary beat frequency (Fig. 6), it is evident that ciliary beat frequency is unresponsive to changes in $[Ca^{2+}]_i$ below 150 nM. In addition, the rate of ciliary beating saturates at $[Ca^{2+}]_i$ above 350–400 nM. At 37°C, an increase in $[Ca^{2+}]_i$ of 175 nm induced the maximum increase in beat frequency, from an increased basal level of ~200 nm to a maximum of ~380 nm (Table 1).

Spatial regulation of ciliary beat frequency by $[Ca^{2+}]_i$

A second important observation made from our simultaneous recordings of $[Ca^{2+}]_i$ and ciliary beat frequency at specific locations was that changes in ciliary beat rate only occurred when the $[Ca^{2+}]_i$ elevation or Ca^{2+} wave reached the location of the cilia within the cell (Figs. 3–5, 7 and 8).

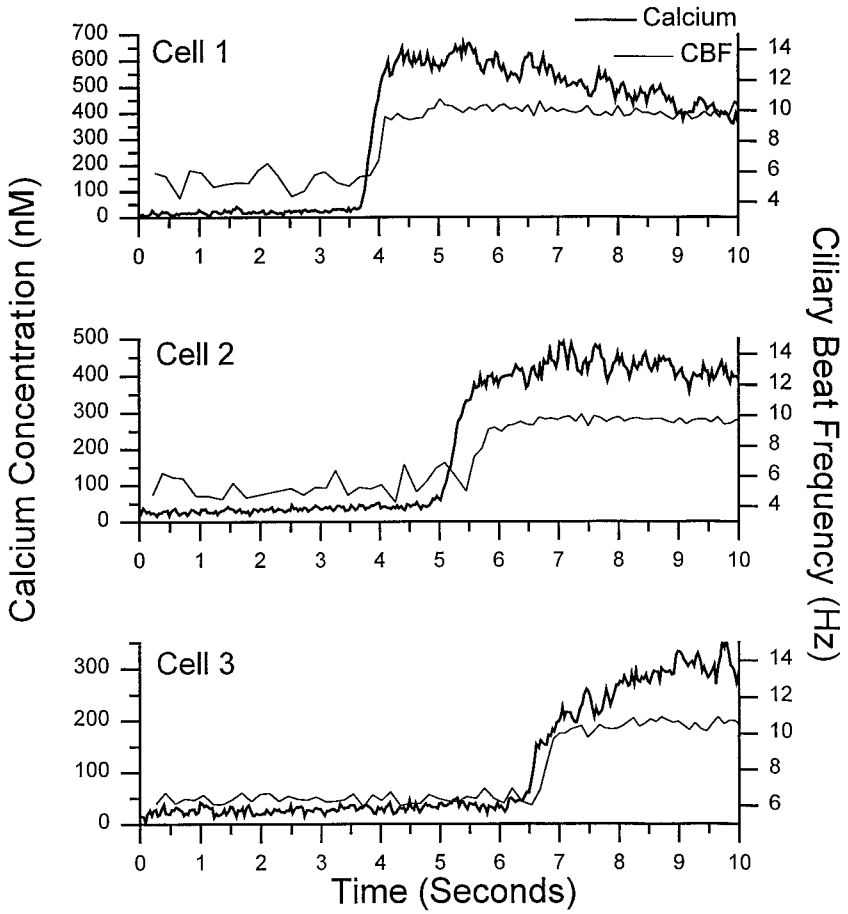

FIGURE 5 The correlation between $[Ca^{2+}]_i$ (*left axis and thick line*) and ciliary beat frequency (*right axis and thin line*) with respect to time for each cell (cells 1–3) shown in Figs. 2–4. Baseline $[Ca^{2+}]_i$ is ~25–50 nM. Baseline ciliary beat frequency is ~4–6 Hz (frequency is calculated from the inverse of each beat period). The $[Ca^{2+}]_i$ is elevated to peak levels ranging from 450 to 650 nM. Beat frequency is also rapidly elevated to a maximum level of ~10 Hz at ~350 nM. Further increases in $[Ca^{2+}]_i$ do not elevate ciliary beat frequency further.

It was commonly observed that, as a Ca^{2+} wave swept across a cell, cilia at one end of a cell increased their beat frequency before cilia at the other end of the cell. Although Ca^{2+} waves at room temperature travel at ~20 μm s^{-1} and take ~½-1 s to cross a cell (Fig. 3) (Sanderson et al., 1990), at 37°C Ca^{2+} waves were measured to travel across cells at ~55 μm s^{-1} (Fig. 8). Even at this rapid rate of wave propagation, a sequential or staggered increase in ciliary activity occurs within individual cells (Figs. 7 and 8). This result, that increases in beat frequency coincide with the propagation of a Ca^{2+} wave front, indicates that increases in beat frequency are the result of a local increase in $[Ca^{2+}]_i$ in the vicinity of the base of the cilium.

DISCUSSION

Previous studies have established that changes in $[Ca^{2+}]_i$ are important for the control of airway ciliary activity. However, an understanding of the Ca^{2+} control mechanism has remained poor, in part, because of an inability to accurately measure the temporal and spatial changes in $[Ca^{2+}]_i$ associated with changes in ciliary beat frequency. The requirements for measuring ciliary beat frequency at multiple sites are the acquisition of images at high speed, with a sampling rate sufficient to resolve the temporal variations in ciliary position, and the analysis of small areas to ensure that recordings pertain to a small group of cilia (Sanderson and Dirksen, 1985; Sanderson et al., 1988). Because cilia can beat at rates of up to 30 Hz (Sanderson and Sleigh, 1981), we have found that an image sample rate of 240 Hz and a sampling area of ~2 μm^2 provide a high-fidelity signal (Sanderson, 1998). The merits of a fast sampling rate have also been emphasized by their ability to measure, in addition to beat frequency, the rest, recovery, and effective phases of the ciliary beat cycle (Sanderson and Dirksen, 1995).

TABLE 1 A correlation between the changes in $[Ca^{2+}]_i$ and ciliary beat frequency induced by intercellular Ca^{2+} waves in airway epithelial cells

	Before stimulation		After stimulation		Induced change		
Temp.	$[Ca^{2+}]_i$	CBF (Hz)	$[Ca^{2+}]_i$	CBF (Hz)	$[Ca^{2+}]_i$	CBF (Hz)	*n*
20–23°C	68 ± 59	6.4 ± 1.3	387 ± 203	11.6 ± 1.8	319 ± 217	5.2 ± 1.7	13
37°C	207 ± 72	17.2 ± 3.2	382 ± 61	26.0 ± 1.7	175 ± 67	8.8 ± 2.7	8

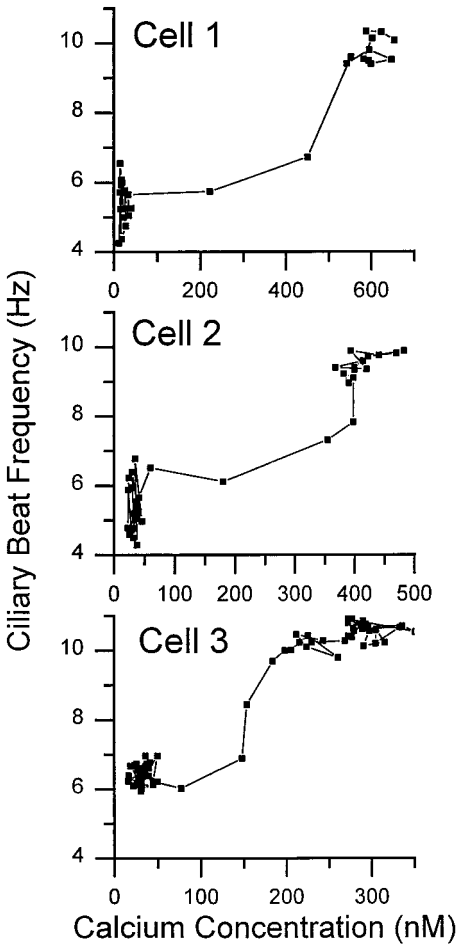

FIGURE 6 The correlation between ciliary beat frequency and $[Ca^{2+}]_i$ for each of the three cells shown in Fig. 5. Ciliary activity changes little with concentrations up to ~150–200 nM. Above 200 nM, ciliary beat frequency rapidly increases and reaches a maximum value at ~350–400 nM. Points are plotted in the form of a scatter plot, but each point is connected by a line that indicates the temporal sequence of the data.

Previously, Korngreen and Priel (1994) and Mao and Wong (1998) simultaneously measured $[Ca^{2+}]_i$ and ciliary beat frequency, and Villalon et al., (1989) and Salathe and Bookman (1995) measured these two parameters separately. Some groups used small detector areas for the measurement of ciliary activity, but all chose to calculate beat frequencies using automated processes based on fast Fourier transforms (FFTs). This approach requires that many data points be collected over one to several seconds. Although average beat frequencies are easily obtained from the peak intensities of the FFTs, the temporal resolution of this approach is low, for both the measurement of the duration of each beat cycle as well as the ability to detect rapid changes in beat frequency.

In this study we have verified that the propagating Ca^{2+} waves are responsible for inducing increases in ciliary beat frequency by simultaneously measuring $[Ca^{2+}]_i$ and beat frequency within the same cell, at the same location. Korngreen and Priel (1994) reported that the increase in ciliary beat frequency was composed of two parts, an initial fast change and a secondary slower change. Our recordings of Ca^{2+}-induced changes in beat frequency do not demonstrate a biphasic response. An explanation for this discrepancy could simply be that the sampling times of overlapping sequential FFTs contain different proportions of the slow and fast beating activities of the cilia. Salathe and Bookman (1995) suggest that the use of FFTs is a convenient alternative to recording data for long-term studies. We suggest that the loss of information outweighs this justification and that, if beat frequencies change rapidly in response to cell stimulation, the rationale for experiments with extended time courses should be reconsidered.

In correlative studies of $[Ca^{2+}]_i$ and beat frequency, the measurement of $[Ca^{2+}]_i$ with high temporal and spatial resolution is equally important because Ca^{2+} signaling in cells is extremely dynamic, consisting of both intracellular and intercellular Ca^{2+} waves (Thomas et al., 1996; Berridge and Dupont, 1994; Sanderson, 1996; Fewtrell, 1993). In addition, the Ca^{2+} signaling machinery of cells, e.g., the IP_3 receptors, ryanodine receptors, and Ca^{2+} stores within the endoplasmic reticulum, are not uniformly distributed throughout cells (Sugiyama et al., 1996; Berridge, 1997), and the responses of individual cells to agonists or stimuli often vary. Because Korngreen and Priel (1994) averaged information from four cells over a period of 2 s and Salathe and Bookman (1995) averaged data from an area 18 × 35 μm (approximately the size of one cell) for periods of up to 2 s with samples generally taken at 10-s intervals, the interpretation of the changes in $[Ca^{2+}]_i$ with respect to ciliary beat frequency is difficult. For example, in young ciliated cells, acetylcholine was reported to invoke Ca^{2+} and beat frequency oscillations with a periodicity of 18.6 s (Salathe et al., 1997). However, the collection of data samples every 6 or 10 s is insufficient to characterize these responses. We demonstrate in this study that a Ca^{2+} wave propagates through three cells in ~3 s at 20–23°C (even faster at 37°C) elevating the beat frequency of each cell sequentially. As a result, we recommend that Ca^{2+} imaging data be collected at 30 images s^{-1}, simultaneously with beat frequency data.

In view of the differences in the technical approaches, it is not surprising that the data collected by different studies are not in full agreement. Salathe and Bookman (1995) suggest that ciliary beat frequency is approximately linearly proportional to the $[Ca^{2+}]_i$, with low beat frequencies of 5 Hz occurring at $[Ca^{2+}]_i$ of 20 nM and higher beat frequencies of ~9 Hz occurring at $[Ca^{2+}]_i$ of 200 nM. In contrast, Korngreen and Priel (1996) report that there is no correlation between beat frequency and Ca^{2+} at low $[Ca^{2+}]_i$ from 70 to 270 nM. Our results indicate that beat frequency is relatively stable at low $[Ca^{2+}]_i$ from 20 to 150 nM, suggesting that basal frequency is inherent to the axoneme. Whereas Salathe and Bookman (1995) did not investigate paradigms that increased the $[Ca^{2+}]_i$ greater than ~200 nM, Korngreen and Priel (1996) elevated $[Ca^{2+}]_i$ in rabbit airway epithelial cells with extracellular ATP to values ap-

proaching 900 nM and reported that ciliary beat frequency was elevated by all $[Ca^{2+}]_i$ up to this level. On the other hand, we have shown that ciliary beat frequency was increased maximally by elevations of $[Ca^{2+}]_i$ to ~350–400 nM.

The mechanism used for increasing $[Ca^{2+}]_i$ may be very important in determining the response of the cilia. We have used the mechanical stimulation of a single cell to generate an intercellular Ca^{2+} wave to elevate $[Ca^{2+}]_i$ in adjacent cells. The mode of wave propagation is believed to be the passage of IP_3 to adjacent cells and the release of Ca^{2+} from intracellular stores (Sanderson et al., 1994). Thus the elevation in $[Ca^{2+}]_i$ is separated from other signaling pathways, such as G-protein-mediated processes or the activation of protein kinase C by diacylglycerol. The direct action of Ca^{2+} at the axoneme rather than at the whole-cell level is also supported by the sequential increases in beat frequency of cilia on the same cell. For example, increases in $[Ca^{2+}]_i$ could activate Ca^{2+}-dependent K^+ channels to induce a change in membrane potential that, in turn, could activate voltage-gated Ca^{2+} channels. This would be expected to result in an almost uniform increase in $[Ca^{2+}]_i$ and a simultaneous increase ciliary beat frequency throughout the cell either by the direct action of Ca^{2+} or by a whole-cell cascade of kinases or phosphates. The use of agonists such as ACh or ATP may activate other transduction pathways (e.g., phosphorylation) that may modulate the effect of Ca^{2+} on the axoneme. For example, Salathe and Bookman (1995) found evidence that ACh was capable of suppressing ciliary beat frequency, even though this agonist commonly increases $[Ca^{2+}]_i$. The direct action of Ca^{2+} on the axoneme is also implied by the findings of Korngreen and Priel (1996), who reported that thapsigargin and ionomycin, which elevate $[Ca^{2+}]_i$ independently of receptor-mediated processes, had similar effects on ciliary beat. The rapid rate at which beat frequency increases after the $[Ca^{2+}]_i$ is increased also suggests that Ca^{2+} is not operating via a complex signaling cascade. Similar propagating changes in

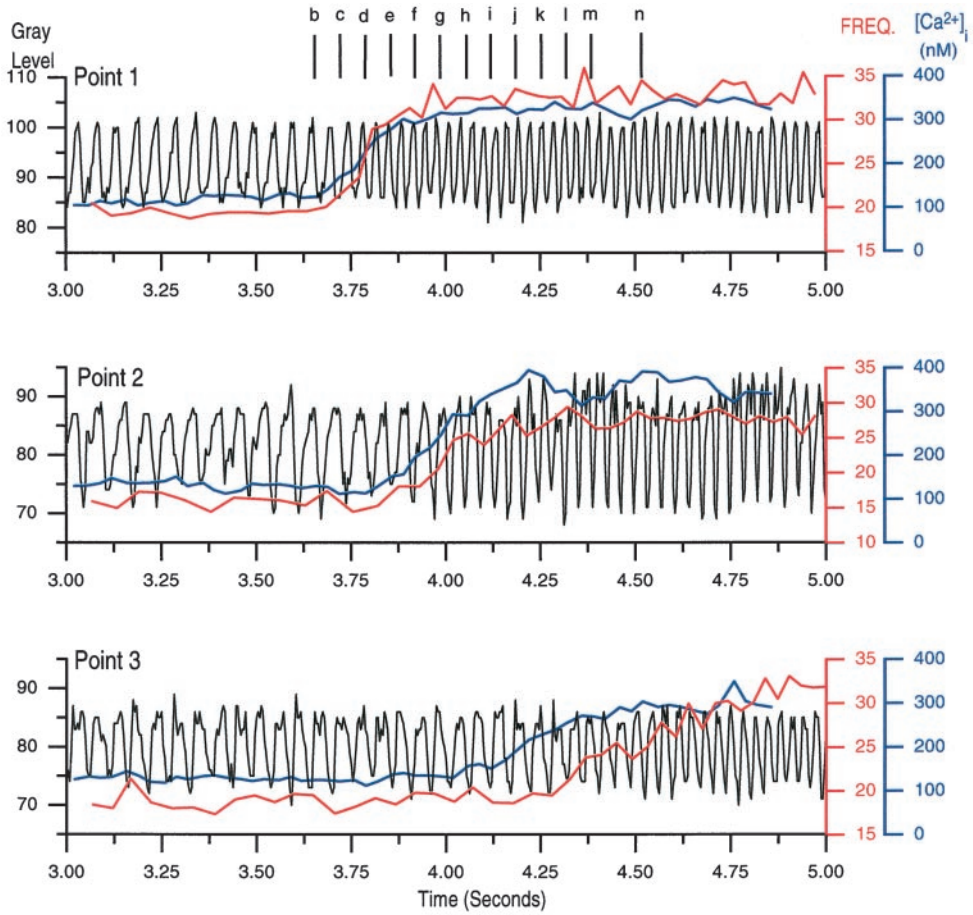

FIGURE 7 The change in ciliary beat frequency induced by increases in $[Ca^{2+}]_i$ within a single cell at 37°C. The ciliary activity for three points (points 1, 2, and 3 are represented in the *top, middle, and bottom traces*, respectively) at different locations within the same cell are represented by the variation image gray level (*left axis, black*) with respect to time. The exact location of the points is shown in Fig. 8 *a*. The analysis of ciliary motion is performed near the middle of the cilia. The ciliary beat frequency is calculated at the end of each beat cycle in each record and superimposed in red (*right axis*). The change of $[Ca^{2+}]_i$ at the base of each group of cilia is superimposed in blue (*right axis*). The exact locations of the corresponding points used to analyze $[Ca^{2+}]_i$ are shown in Fig. 8 *a*. The $[Ca^{2+}]_i$ rises in sequence at the three points within the cell as the Ca^{2+} wave propagates across the cell. The ciliary beat frequency increases just after the rise in $[Ca^{2+}]_i$ to rapidly reach a maximum level. The vertical lines (*top trace*) correspond to the image panels in Fig. 8.

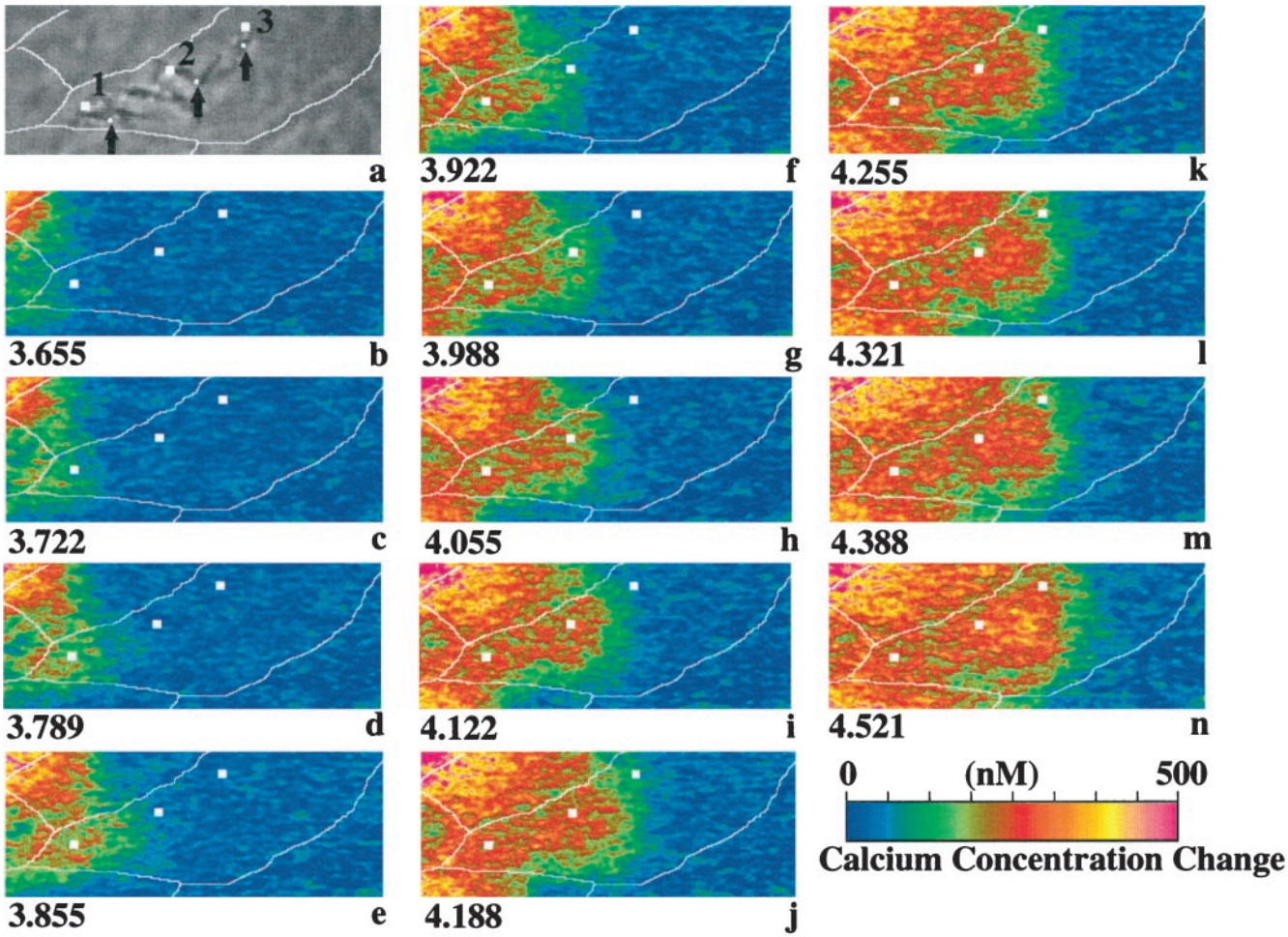

FIGURE 8 A series of sequential images (*b–n*) showing the propagation of an intercellular Ca^{2+} wave across the individual cell (*a*), described in Fig. 7, from which ciliary beat frequency was recorded. The Ca^{2+} wave enters the cell at the lower left and sweeps along the upper edge and across the cell. The white lines indicate the estimated position of the cell boundaries. (*a*) The phase-contrast image of the cell shows the location from which ciliary beat activity is recorded (*small white squares with black arrows*). In addition, the image shows the position, at the base of each respective group of cilia (*white square with black number*), where the $[Ca^{2+}]_i$ was measured. The time of each image (in seconds) is indicated at the lower left of each image. The relationship of the images to the time traces shown in Fig. 7 are indicated by the vertical lines. The lettering indicates the corresponding image. The $[Ca^{2+}]_i$ shown in each image, represented by the color scale, indicates the change in $[Ca^{2+}]_i$ rather than absolute $[Ca^{2+}]_i$ and was calculated by subtracting the initial $[Ca^{2+}]_i$ at the onset of the experiment from the $[Ca^{2+}]_i$ during the experiment.

$[Ca^{2+}]_i$, although induced by a different mechanism, induced a rapid ciliary arrest of the gill cilia of *Mytilus* (Motokawa and Satir, 1975). In this case, the cilia appeared only to respond to Ca^{2+} at the end of the recovery stroke. This raises the possibility that increases in beat frequency of airway cilia may only be initiated when the cilium reaches a certain stage of the beat cycle. However, this does not seem to be the case, because the increase in beat frequency occurred over several beat cycles.

In conclusion, high-resolution recording techniques have revealed that ciliary beat frequency changes can occur very rapidly in response to changes in $[Ca^{2+}]_i$, and that maximum increases in beat frequency are induced by relatively small changes in $[Ca^{2+}]_i$.

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