

Mechanosensitivity of cultured ciliated cells from the mammalian respiratory tract: Implications for the regulation of mucociliary transport

(cilia/beat frequency/calcium/mucociliary clearance)

MICHAEL J. SANDERSON AND ELLEN R. DIRKSEN

Department of Anatomy, UCLA School of Medicine, University of California at Los Angeles, Los Angeles, CA 90024

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ABSTRACT Mechanical stimulation of the cell surface or cilia of cultured ciliated epithelial cells derived from the rabbit tracheal mucosa resulted in a transitory ciliary beat frequency increase of 20% or more. This response was composed of a lag, rise, and recovery phase. The duration of the response, but not the maximal frequency, was increased by stronger stimulation. The ability of these ciliated cells to respond to stimulation depended on culture age; generally, cultures younger than 4 days were insensitive. The frequency response was transmitted to adjacent and more distal cells in all directions. The lag phase but not the rise time of the adjacent cell response was extended. These ciliary responses were lost when extracellular Ca^{2+} was removed. Replacement of Ca^{2+} resulted in a restoration of mechanosensitivity. In 1 mM verapamil and Ca^{2+} the frequency response was also lost. These results suggest that the frequency response is dependent on Ca^{2+} influx (although its intracellular action is unknown) and imply that mucociliary clearance is a localized self-regulating process.

Mucociliary clearance is an important lung defense mechanism preventing the accumulation of inhaled contaminants within the respiratory tract (RT) (1, 2). The role of cilia in mucus transport is vital. By providing the driving force, the cilia can determine and control most of the characteristics of mucus transport. However, the mechanisms by which RT ciliated cells regulate mucus transport by means of their cilia are unknown (1, 2).

If a process is to be regulated by cilia, it is essential that ciliary activity be adjusted in response to varying environmental conditions. A relevant example would be a change in mucus load. In non-mammalian ciliated systems environmental information is frequently transduced into changes in ciliary activity by Ca^{2+} -dependent mechanosensitivity. In *Paramecium* and *Stylonychia* physical stimulation of the anterior cell surface, usually arising from collision during normal movement, results in the reversal of the ciliary beat pattern and a concurrent increase in beat frequency. This response is induced by a membrane depolarization and an elevation of intracellular Ca^{2+} (3, 4). Similarly, tactile stimulation leading to elevated intracellular Ca^{2+} has been found to increase, but not reverse, the beat frequency in salamander oviduct (5), frog palate (6), and *Mytilus* abfrontal (7) cilia.

The mechanism by which Ca^{2+} mediates these changes is not fully understood; evidence suggests that Ca^{2+} acts in conjunction with calmodulin at an axonemal site (8). In salamander ciliated oviduct cells Ca^{2+} is thought to modify cell ATP metabolism (5). A role for Ca^{2+} in the control of mammalian ciliary activity has been proposed (9-11). Elevated intracellular Ca^{2+} , induced with ionophore A23187 in intact cells and Ca^{2+} -calmodulin complexes added to deter-

gent-permeabilized ciliated cell models (9-11), has been shown to increase beat frequency without altering beat orientation of RT and oviduct cilia.

We report here that mammalian RT ciliated cells are also sensitive to mechanical stimulation in a Ca^{2+} -dependent manner and that this sensitivity can be communicated to adjacent cells. We believe these responses are fundamental to the regulation of mucociliary clearance in view of the mechanical interactions that occur between cilia and mucus.

MATERIALS AND METHODS

Details of the cell culture technique and the data acquisition system have been described elsewhere (12). Cultures of ciliated cells were grown from explants of mucosal tissue, isolated from the trachea of rabbits, on collagen-coated coverslips. Before use each culture was washed at room temperature with, and then mounted in, Hanks' balanced salt solution (HBSS) that was modified by increasing CaCl_2 to 2 mM, adding 1 mM EGTA and 25 mM Hepes, omitting NaHCO_3 , and reducing NaCl to 81 mM. For Ca^{2+} -free conditions cultures were washed twice for 5 min in HBSS without CaCl_2 . When required, 2 mM MnCl_2 was substituted for CaCl_2 . Verapamil was dissolved in ethanol and added to HBSS to a concentration of 1 mM verapamil and 1% ethanol. For observation, cultures were transferred to a temperature-regulated holder (usually 20-25°C) and viewed with phase-contrast optics ($\times 40$ water-immersion lens). The movement of the cilia was detected and transduced by a phototransistor into a photoelectronic signal that was recorded by a computer-assisted data acquisition system. Accurate alignment of the phototransistor over the cilia was achieved with the aid of cross-hair sights (Fig. 1).

Mechanical Stimulation. The cilia of the cell to be stimulated were aligned so that a repetitive photoelectronic signal was recorded. A glass microprobe (tip diameter $\approx 1 \mu\text{m}$), attached to a piezoelectric device, was brought into alignment with a micromanipulator over the selected cell adjacent to the cilia. The cell was stimulated by dimpling its surface with a vertical movement of the microprobe. Stimulation of the cilia was achieved by rotating the piezoelectric device through 90° to provide a horizontal pulse. The displacement pulse of the probe was driven by a single square-wave voltage pulse lasting 175 msec. Tip displacement was linearly proportional to the applied voltage: $0.464 \mu\text{m}/\text{V}$. The stimulating pulse was recorded by the data acquisition system.

The mechanosensitive response of RT ciliated cells consists of a lag, rise, and recovery phase. The lag phase is the time elapsed before the beat frequency increases following the termination of stimulation. A measure of the rise and recovery phases is provided by the rise and recovery times, respectively. The rise time is the time taken (from the end of

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Abbreviation: RT, respiratory tract.

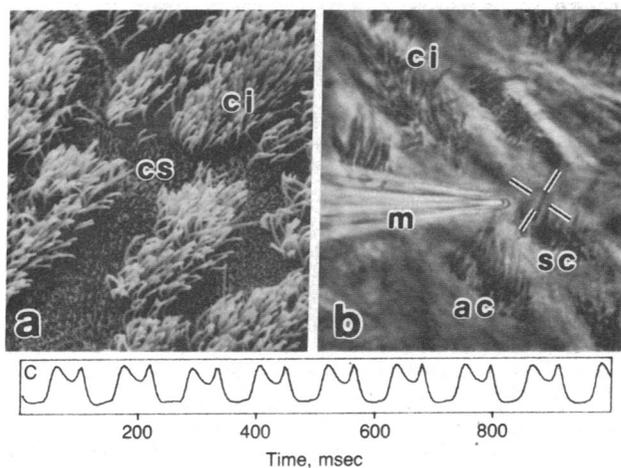


FIG. 1. Scanning electron ($\times 1250$) (a) and light ($\times 750$) (b) micrographs of cultured rabbit tracheal ciliated cells. Cilia (ci) of the cell to be stimulated (sc) or the adjacent cell (ac) were aligned with the photodetector with the aid of the cross hairs. The microprobe (m) stimulated the cell surface (cs). (c) The recorded photoelectronic signal shows the triphasic nature of the beat cycle; left to right, the trough, first peak, and second peak represent the rest phase, recovery phase, and effective phase, respectively.

the lag phase) for the frequency to increase by half the final frequency increase. The final frequency increase is determined by subtracting the initial frequency from the maximal frequency achieved during the response. The recovery time is the time taken (from the time the frequency initially reaches its maximal level) for the beat frequency to be reduced by half the final frequency increase.

RESULTS

We observed that ciliary beat frequency of RT cells increased as surrounding fluids were exchanged. The direct stimulation of a cell by dimpling its surface with a microprobe (Fig. 1) confirmed that cultured RT ciliated cells respond to mechanical disturbance by rapidly elevating the beat frequency of their cilia (Fig. 2). The expression of this mechanosensitivity is, however, influenced by culture age (Fig. 2c). A frequency response could also be induced by the direct stimulation of the cilia with the microprobe or by drawing mucus attached to the microprobe over the cilia. Mechanosensitivity was displayed by the cells that remained in the original explant.

Influence of Tissue Culture Age on Mechanosensitivity. Cell cultures were suitable, as judged by the morphology and extent of the cellular outgrowth from the explant, for experimentation after 3 days. At this stage the cells were found to lack mechanosensitivity. A range of increasing stimuli was given to ensure that a threshold or stronger stimulus was delivered. Most cells were still unresponsive after 5 days of culture. However, a weak response was obtained in a few cases when a large microprobe displacement ($>10 \mu\text{m}$) was applied. In addition, many insensitive cells could be forced to elicit a short response if manually stimulated. Cell damage frequently accompanied these stimulations. This activation was followed by ciliary arrest, suggesting the stimulus was lethal. After 6 or 7 days in culture almost all of the cells displayed mechanosensitivity in response to stimuli $<10 \mu\text{m}$ (Fig. 2c). The development of sensitivity correlated with cell morphology. In young cultures the boundaries of individual cells are not easily distinguishable. The cilia of adjacent cells are closely associated and the culture has a multilayered appearance, reflecting the organization of the original epithelium. In older cultures the boundaries of cells are easily recognized as the cells flatten to form a monolayer.

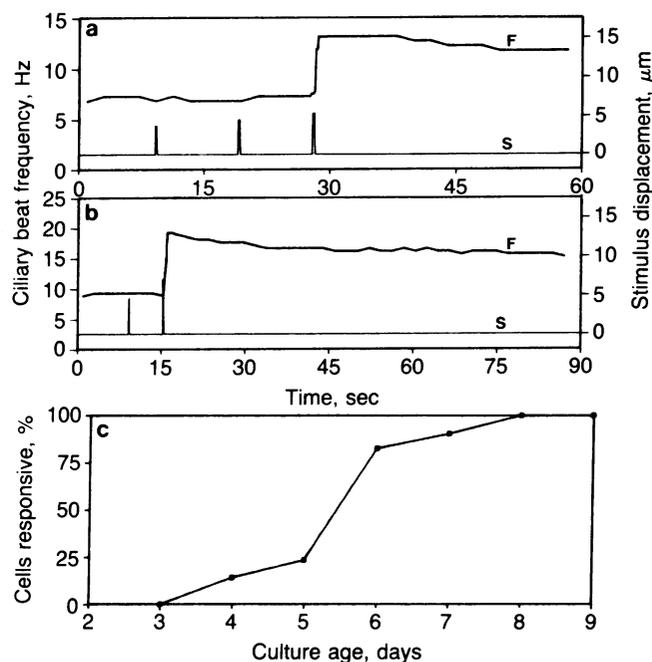


FIG. 2. Frequency response (F) of RT ciliated cells to a range of stimuli (S). (a) A 10-day-old culture at 25°C . (b) A 5-day-old culture at 40°C . (a and b) The beat frequency was calculated by a fast Fourier transform of the original signal every 2.048 sec (512 points \times 4 msec), except in the vicinity of the stimulus pulse. In this region frequency was calculated from the individual beat cycles (see Fig. 5). (c) Summary graph showing the redevelopment of mechanosensitivity of ciliated cells with respect to culture age.

Frequency Response Characteristics. At room temperature in HBSS the cilia of undisturbed cultured ciliated cells maintain a beat frequency (3–10 Hz) that does not vary with culture age. The mean beat frequency at 3, 5, and 7 days was 6.77, 6.74, and 6.25 Hz, respectively. The minimal stimulus required to elicit a response was variable, which may result from the difficulties of accurately aligning the microprobe with the irregular topography of the ciliated cells in culture rather than being a reflection of cell sensitivities. To overcome these positional errors the threshold stimulus intensity was determined in a relative manner. By maintaining a small incremental step, a measurement of the intensity of the stimulus can be obtained by calculating the tip displacement between two successive stimuli in which the first stimulus did not, but the second did, invoke a response (Figs. 2 and 3). With this approach, a stimulus consisting of a $1\text{-}\mu\text{m}$ tip displacement for 175 msec invoked a frequency response.

The effect of stronger stimuli on the response was determined by initially obtaining the threshold stimulus for a cell, after which stronger stimuli of known magnitude were applied. Under these conditions the maximal frequency reached during a response did not increase with stronger stimulation (Fig. 3). Although the maximal frequency attained during each response is consistent for an individual cell, irrespective of the stimulus intensity, the maximal frequencies reached varied between cells. The ciliary beat frequency of most cells increased by 20–60% but, in some cases, reached frequencies greater than double the original rate (Fig. 4). Under warmer conditions ($36\text{--}39^{\circ}\text{C}$) similar percentage increases were observed, even though the initial beat frequencies of the cilia were higher (Figs. 2b and 3d). In contrast to the maximal frequency of the response, the duration of the response increased following stronger stimulation (Fig. 3). After threshold stimulation the elevated beat frequency returned toward its original level, but the onset and rate of frequency rebound were delayed by stronger stimulation. In experi-

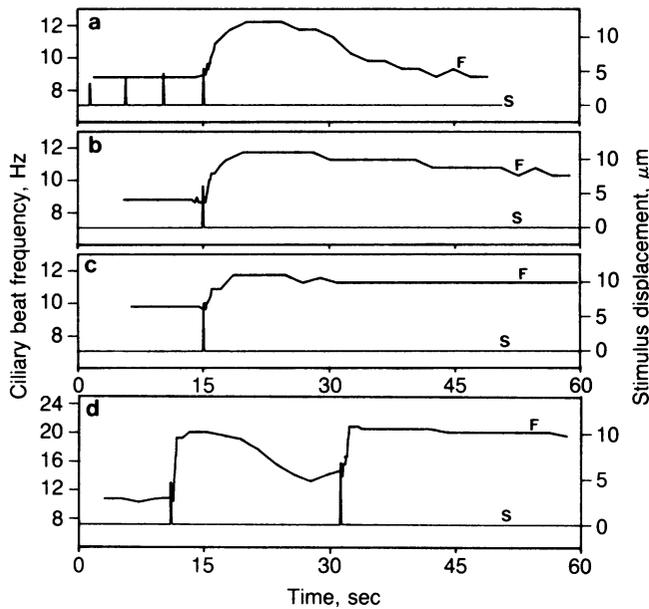


FIG. 3. Effect of increasing stimulus magnitude on mechanosensitivity. (a) The threshold stimulus was found by applying successive incremental stimuli (S). (b and c) Subsequent stronger stimuli to the same cell invoked responses with similar maximal frequencies, but with extended recovery times. (d) A threshold and an extended response demonstrated by a single cell, induced by two successive stimuli, at 40°C. The maximal frequency reached in each response was similar, but the duration of the second response was increased.

ments in which a pulse between 4.6 and 8.9 μm was applied, 75% of the cells displayed a response with a recovery time of >45 sec. However, because of inaccuracies of microprobe positioning, some of the stimuli were probably weaker. Thus, the remaining 25% of the cells displayed a response with a shorter recovery time that ranged from 12 to 28 sec (mean = 19.34, $n = 12$).

The development of the frequency response consists of two stages: an initial lag phase, during which the beat frequency is unaltered, and a rise phase, during which frequency increases (Fig. 5). In the stimulated cell the lag phase (mean = 285 msec, $n = 11$, SD = 190) and rise time (mean = 234 msec, $n = 11$, SD = 110) were similar. At warmer temperatures (38°C), the lag phase (mean = 157 msec, $n = 9$, SD = 110) and rise time (mean = 188 msec, $n = 9$, SD = 96) were similarly shortened.

Cellular Communication of Stimulation. The frequency response was transmitted to the surrounding cells in all directions. However, the lag phase of the immediately adjacent cells was extended (mean = 3.225 sec, $n = 9$, SD = 1.1), whereas the rise time of the response was similar (mean = 348 msec, $n = 8$, SD = 160) (Fig. 5). The lag phase of the

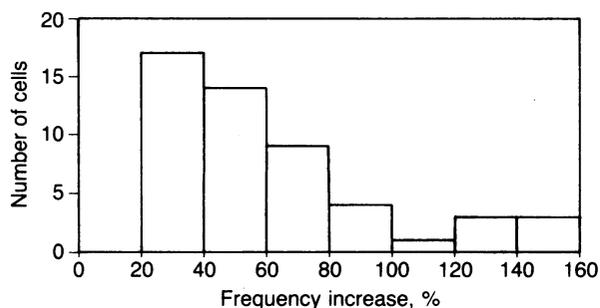


FIG. 4. Variation in the magnitude (%) of the frequency increases of individual cells following stimulation.

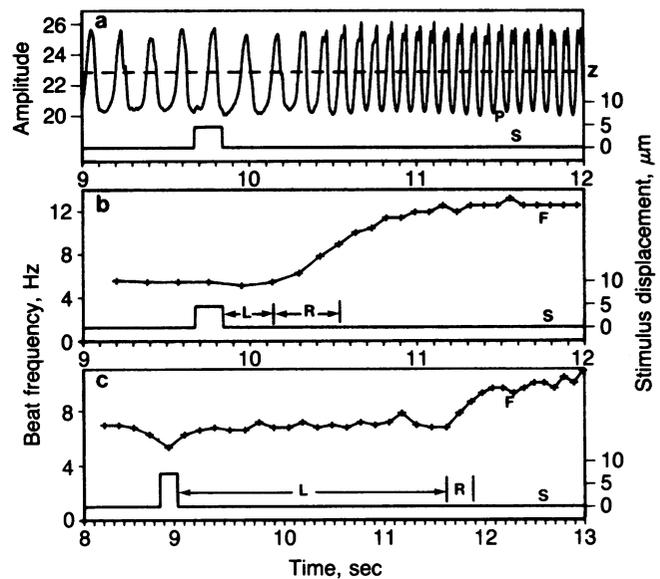


FIG. 5. Beat frequency (F), calculated from the signal period measured at Z, of the photoelectronic signal (P), is plotted for the stimulated (b) and adjacent (c) cell to provide a detailed frequency response immediately following stimulation (S). Beat frequency increases after a lag phase (L) with a rise time (R). The lag phase of the adjacent cell is longer than that of the stimulated cell. In a the amplitude of the original signal remains relatively constant. The shape of the waveform changes slightly due to a small change in the position of the cilia relative to the sensor.

next neighboring cell was further extended (mean = 4.002 sec, $n = 8$, SD = 1), again, without affecting the rise time (mean = 340 msec, $n = 8$, SD = 171). The range of the responses of the adjacent cells was indistinguishable from the stimulated cell responses. Although the response was transmitted across many cells, its transmission faded with distance. The neighboring cell response (mean = 18.96 sec, $n = 8$, SD = 5.96) was shorter than the stimulated cell response. However, two cells had recovery times of >35 sec. The second neighboring cell also had a short recovery time (mean = 15.52 sec, $n = 7$, SD = 8.57), although, again, two cells had recovery times of >35 sec. As a result, a concentric wave of increased activity originating from the stimulated cell spread out over the culture. The original activity was then restored in a reverse manner. Detection of stimulation and transmission of a frequency response was not only limited to ciliated cells; the stimulation of a non-ciliated cell also resulted in adjacent ciliated cells displaying a delayed response.

Calcium-Dependent Mechanosensitivity. Mechanosensitivity was lost when extracellular Ca^{2+} was removed (Fig. 6). After 20 min without extracellular Ca^{2+} , the cilia continued to beat with a frequency similar to that observed in the presence of Ca^{2+} . Stimulation of the cell did not lead to the expected increase in frequency but, instead, resulted in a decline in ciliary activity. (The beat frequency of the adjacent cells was unaltered.) This decline in ciliary activity occurred because of a simultaneous reduction in beat frequency and amplitude. The beat amplitude was severely reduced to a vibrational motion of the cilia at the end of their effective stroke, although the frequency was only moderately reduced. However, since the cilia were no longer completing full beat cycles, signal frequency was no longer considered equivalent to beat frequency, although it still accurately recorded the vibrational oscillations of the cilia (Fig. 6). When Mn^{2+} was added to Ca^{2+} -free HBSS the mechanosensitive response did not occur following stimulation, but the decline in ciliary activity was prevented. Under these conditions the cilia continued to maintain a beat frequency. The replacement of

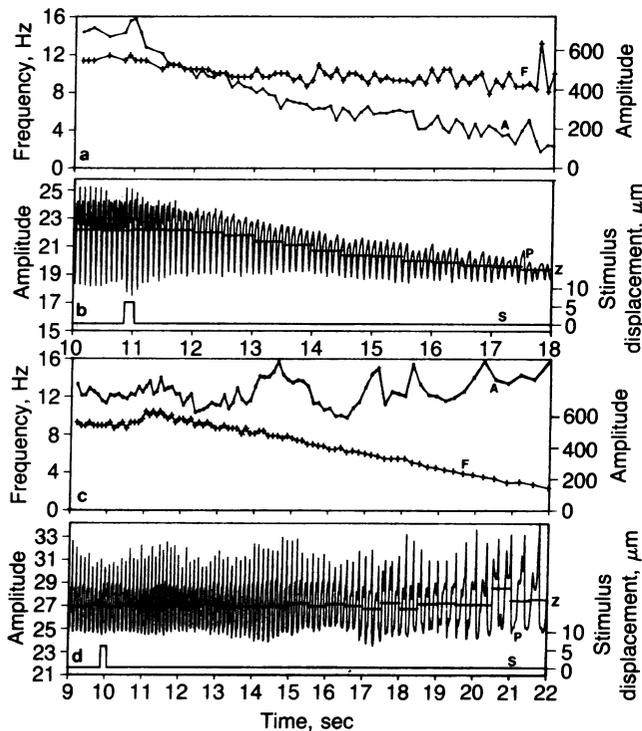


FIG. 6. (a and b) In Ca^{2+} -free conditions, the frequency (F) response to stimulation (S) was lost; the photoelectronic signal (P) rapidly declined in amplitude (A) and lost its triphasic form. (c and d) In the presence of Ca^{2+} and verapamil, stimulation produced a transient increase in frequency that was followed by a decline. The amplitude and various phases of the photoelectronic signal were maintained during this decline. Frequency was calculated from the signal period measured at the mean signal amplitude (Z).

Ca^{2+} and removal of Mn^{2+} by washing with complete HBSS restored mechanosensitivity to the stimulated and adjacent cells.

The prestimulus frequency of the ciliated cells was unaffected by the presence of either 100 μM or 1 mM verapamil. Similarly, the frequency response of the stimulated or the adjacent cells was apparently unaltered in the presence of 100 μM verapamil. However, increasing the verapamil concentration to 1 mM greatly reduced the frequency response to a small, transient increase. This was also followed by a decline in ciliary activity leading to arrest (Fig. 6), which resulted from a reduction in beat frequency but not in amplitude. In 3 of 14 cases a frequency reduction was not observed and in 8 of 10 cases the adjacent cell showed no change in activity. The presence of 1% ethanol did not affect the cellular responses.

DISCUSSION

Cultured ciliated cells from the RT of rabbits are capable, at both room and body temperature, of quickly responding to mechanical disturbances by temporarily increasing their ciliary beat frequency. In culture, RT cells are devoid of any innervation; consequently, mechanosensitivity must be unequivocally associated with the ciliated cell. The ability of the RT cells to respond to stimulation was lost when extracellular Ca^{2+} was removed. This contrasts with the ciliary activity of cultured ciliated cells from the mammalian oviduct. In the absence of extracellular Ca^{2+} , these cells can increase their ciliary beat frequency in response to prostaglandin stimulation by releasing calcium from intracellular stores (9). The frequency increase of RT cells was also blocked by verapamil, a calcium channel blocker (13), in the presence of

Ca^{2+} . Collectively, this evidence suggests that the mechanosensitivity of RT ciliated cells is dependent on extracellular Ca^{2+} that moves across the cell membrane by means of calcium channels, although the intracellular mechanism of Ca^{2+} action is unknown. For technical reasons only a few of our experiments were performed at body temperatures (36–39°C). However, the similarity of the mechanosensitive response at different temperatures suggests that the basic description formulated in this study at 20–25°C will be generally applicable at the higher temperatures.

A similar Ca^{2+} -dependent transduction mechanism has been described in *Paramecium* and *Stylonychia*. In these protozoa, mechanosensitive channels are located in the non-ciliary cell membrane (3, 4, 14, 15). The mechanosensitive site of cultured RT ciliated cells could also be located in the somatic cell membrane rather than in the ciliary membrane. Although stimulation of the cilia, either directly or with mucus, produced a frequency response, this evidence does not exclusively support the idea that the transduction site is located on the ciliary membrane. It is possible that such stimulation was transmitted, by means of the cilium, to the somatic membrane for transduction. Similar suggestions have been made for *Paramecium* (4) and mollusc sensory cilia (16). Furthermore, the transduction of mechanosensitivity by RT cells does not appear to use properties specifically associated with the ciliary membrane since the stimulation of non-ciliated cells produced frequency responses in adjacent ciliated cells. The localization of the mechanosensitive site in the somatic membrane has practical implications. In this location the sensor would not be perturbed by the normal activity of the cilium but would be ideally placed to detect mechanical stresses occurring at the ciliary base as the cilium engages the overlying mucus.

The location of a mechanosensitive channel in the cell membrane may also explain the loss and reexpression of mechanosensitivity as the ciliated cells grow in culture. Young cultures, insensitive to normal stimulation, will respond to heavy stimulation that often results in membrane rupture. Cell membrane damage would allow the axoneme to be exposed to extracellular ions. Thus, the axoneme retains the capacity to produce a response in the early stages of culture, suggesting that sensitivity is a membrane-associated phenomenon. Since these cells appear to migrate from the explant (12, 17) while retaining their cilia, we suggest that the properties of the somatic membrane are altered during the early culture periods in a manner incompatible with stimulus transduction. Once migration stops, as in older cultures, the membrane regains its stimulus transduction properties.

Compared to the rapid mechanosensitive responses of *Paramecium* (3, 14), *Stylonychia* (4), and the ciliated cells of *Mytilus* (7, 18) that reach a maximum in <25 msec, the response of cultured RT ciliated cells is slow. This suggests that although the mechanosensitivity of RT cilia is dependent on Ca^{2+} influx across the cell membrane, the transduction process does not involve a ciliary membrane amplification mechanism dependent on voltage-sensitive channels. It is possible that, in the absence of voltage-dependent channels, this delayed time course may be explained by a slow accumulation of sufficient Ca^{2+} in the axoneme to induce a response (19). The alternative hypothesis, that entering Ca^{2+} does not directly mediate the axonemal response but instead initiates the production or release of a second intracellular messenger, is more attractive. A similar conclusion has been proposed for the delayed mechanosensitivity of salamander cilia (5). The existence of a Ca^{2+} -induced calcium release, as found in other excitable cells (20), would be consistent with the effects of Ca^{2+} on the frequency of demembrated mammalian cilia (10, 11). Further evidence for a second intracellular messenger is provided by the adjacent cell responses. Communication of the mechanosensitive arrest

response of *Mytilus* lateral cilia, from the stimulated to the adjacent cell, occurs very rapidly by electrical coupling (18). The response spreads across many cells in <100 msec. In RT ciliated cells the response takes ≈ 3 sec to be transmitted across one cell boundary—a duration too slow for communication by electrical coupling. A simpler explanation for this phenomenon could be provided by assuming that an intracellular messenger is being transmitted by means of gap junctions. Numerous, small intercellular connections, identified as gap junctions, have been demonstrated in RT epithelial cells of guinea pigs (21). The existence of a second intracellular messenger remains to be established.

The ciliary responses in the presence of verapamil and Ca^{2+} or under Ca^{2+} -free conditions may be collectively explained in terms of intracellular calcium concentration (7). Elevated intracellular Ca^{2+} , induced by stimulation and Ca^{2+} channel opening, invokes a frequency response. The response is probably rectified by restoring the cytoplasmic Ca^{2+} to normal. When blocked by verapamil only a small increase in frequency is observed; this is compatible with the use-dependent characteristics of the drug (13). The subsequent decrease in frequency may be explained by a reduction to below normal levels of cytoplasmic Ca^{2+} . This would be due to an imbalance between the removal of Ca^{2+} by means of pumps to the exterior or to internal sequestering sites and the normal inward flow by means of blocked channels (22). This imbalance would be exaggerated if the small amount of Ca^{2+} entering the cell was capable of elevating sequestering rates. In Ca^{2+} -free conditions opening of membrane channels may allow Ca^{2+} to flow out of the cell, severely depleting cytoplasmic Ca^{2+} and inducing abnormal and reduced ciliary activity. A Ca^{2+} loss from the cell is supported by evidence that when Mn^{2+} , a calcium channel antagonist, is present, the reduction of activity is not observed.

The concentration of verapamil used in this study may be high, but low concentrations of the drug do not always completely block calcium influx into the cell (13). Consequently, Ca^{2+} -dependent processes may still occur, especially those that are mediated by the production of a second intracellular messenger. Therefore, it seems reasonable that relatively high doses of the drug would be required to abolish mechanosensitivity. Drug toxicity does not appear to be a problem since nonstimulated cells maintain their beat frequency in the presence of verapamil.

We believe that the possession of mechanosensitivity by RT ciliated cells is a fundamental regulatory mechanism of mucociliary clearance. The implication of our hypothesis is that mucociliary clearance is a localized, self-regulating process, a suggestion that fits well with the sparse motor innervation of the RT epithelium. Contact between cilia and mucus results in local, mechanical stimulation leading to the elevation of beat frequency in the immediate vicinity. Furthermore, cellular communication of the response locally coordinates a cooperative increase in activity of the cilia of adjacent cells to transport the overlying mucus. Mucociliary clearance, occurring on isolated frog palate, also appears to display cooperative ciliary activation. In the absence of intact innervation, mechanical stimulation of the palate increases ciliary beat frequency. In addition, the beat frequency of cilia

at a specific point increases and then declines as particles embedded in mucus approach and move away from that point (23). In our studies, the mechanosensitive response is observed as a frequency increase in the absence of mucus. However, in the presence of overlying mucus, the frequency response would probably be expressed by an increase or maintenance of the work performed, in order to move the extra load. A similar phenomenon of autoregulation of ciliary beat frequency with increasing viscous loads has been demonstrated in RT (24) and newt lung (25) ciliated cells. However, the role of calcium in autoregulation has not been determined.

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