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Mechanical stimulation and intercellular communication increases intracellular Ca^{2+} in epithelial cells

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Intercellular communication of epithelial cells was examined by measuring changes in intracellular calcium concentration ($[Ca^{2+}]_i$). Mechanical stimulation of respiratory tract ciliated cells in culture induced a wave of increasing Ca^{2+} that spread, cell by cell, from the stimulated cell to neighboring cells. The communication of these Ca^{2+} waves between cells was restricted or blocked by halothane, an anesthetic known to uncouple cells. In the absence of extracellular Ca^{2+}, the mechanically stimulated cell showed no change or a decrease in $[Ca^{2+}]_i$, whereas $[Ca^{2+}]_i$ increased in neighboring cells. Iontophoretic injection of inositol 1,4,5-trisphosphate (IP_3) evoked a communicated Ca^{2+} response that was similar to that produced by mechanical stimulation. These results support the hypothesis that IP_3 acts as a cellular messenger that mediates communication through gap junctions between ciliated epithelial cells.

Introduction

Intercellular communication enables cells to coordinate their physiological activity and establish cellular cooperation. This imparts to cellular systems the ability to respond uniformly to a localized stimulus—a response important in cellular behavior and differentiation (Loewenstein, 1981; Caveney, 1985; Spray and Bennett, 1985; Fraser *et al.* 1987). An example of a cooperative cell system is the ciliated epithelium of the mammalian respiratory tract. The function of the ciliated cells in this epithelium is the transportation and expulsion of mucus and debris from the airways of the lungs, a process that requires the collective action of many individual cilia across contiguous cells (Sanderson and Sleigh, 1981). In previous studies we discovered that mechanical stimulation of a respiratory tract ciliated cell in culture induced an acceleration in ciliary beat frequency not only of the stimulated cell, but also of neighboring cells (Sanderson and Dirksen, 1986; Sanderson *et al.*, 1988). Each cell responded with an increase in beat frequency after a lag time that was proportional to the distance of the cell from the stimulated cell. These increases in beat frequency appeared to be mediated by increases in intracellular calcium concentration ($[Ca^{2+}]_i$) (Sanderson and Dirksen, 1988; Sanderson *et al.*, 1989). Thus, mechanosensitivity provides airway epithelial cells with a physiological mechanism for cellular cooperation. Interactions between mucus and cilia could lead to the mechanical stimulation of the cell; the resulting increase in ciliary beat frequency, in both the stimulated and surrounding cells, would provide a local increase in the driving force precisely at the site of the mucus load.

Although stimulus transduction followed by intercellular communication is a fundamental paradigm in cell biology, the mechanism by which airway and other epithelial cells communicate is not well understood. Changes in $[Ca^{2+}]_i$ play a key role in the responses of respiratory tract epithelial cells. Consequently, we have measured the temporal and spatial changes in $[Ca^{2+}]_i$ that occur in these cells in response to mechanical stimulation to explore the mechanisms that mediate integrated cellular activity.

Results

Respiratory tract epithelial cells used in this study were grown in culture for 7–10 d. The cilia and boundaries of these cells in culture are easily identifiable (Figure 1). The $[Ca^{2+}]_i$ of these cells was measured before and after mechanical stimulation with the use of digital video fluorescence microscopy and the Ca^{2+}-specific dye

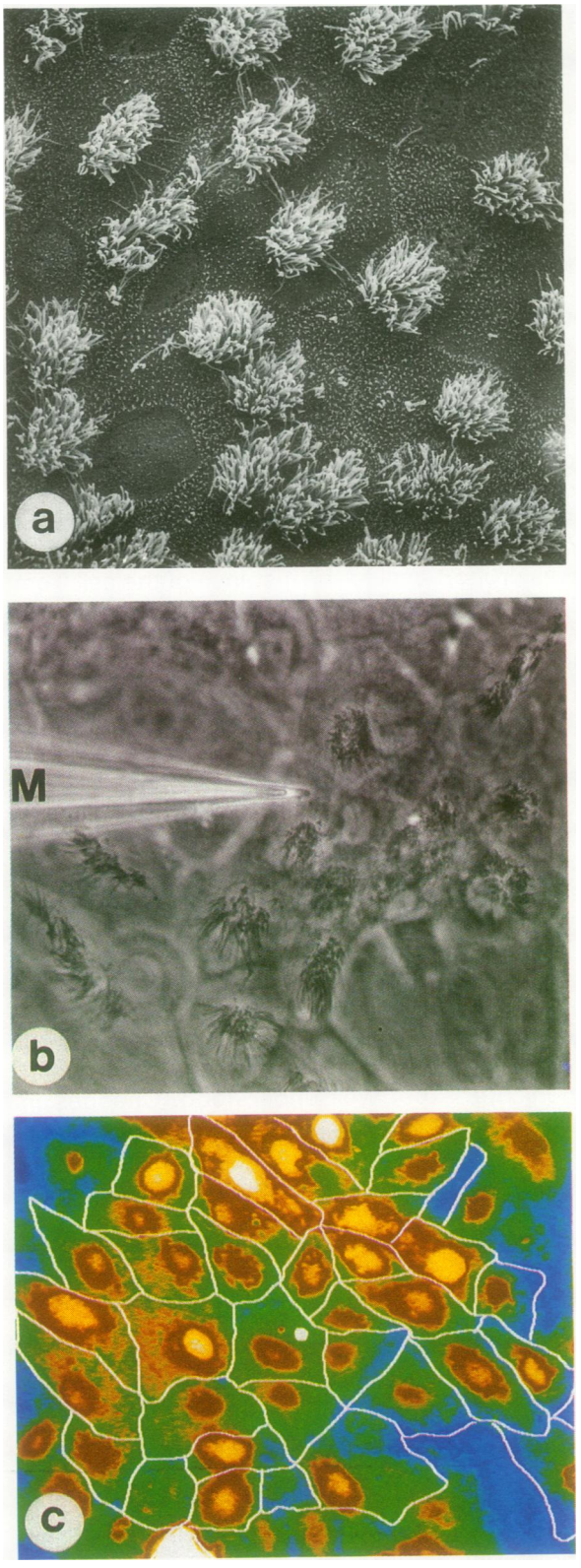

Figure 1. **Cultured respiratory tract epithelial (both ciliated and nonciliated) cells observed with (a) scanning electron microscopy (×1600), (b) phase-contrast light microscopy (×500), and (c) fluorescence microscopy after loading the cells with fura-2 (×400).** (a) The cilia of each cell are centrally located. The cell borders are clearly distinguishable by both scanning electron microscopy and (b) light microscopy. A microprobe (M) is aligned over an individual cell for stimulation. (c) Cells loaded with fura-2 and illuminated with ultraviolet light (380 nm) fluoresce brightly. This image has been pseudocolored for the representation of fluorescence intensity. The cell outlines have been highlighted in white. The cell area shown in (c) is the same as that illustrated in Figure 2 but before any stimulation took place.

fura-2 (Grynkiewicz *et al.*, 1985; Williams *et al.*, 1985) (Figure 1c). Changes in fluorescence intensity were recorded primarily at a single wavelength (380 nm); conventional image pairs at 340 and 380 nm were also recorded to act as calibration points for the single-wavelength recordings.

Mechanical stimulation elevates $[Ca^{2+}]_i$

The effect of mechanical stimulation on the absolute and relative changes in $[Ca^{2+}]_i$ of cultured epithelial cells in the presence of extracellular Ca^{2+} is mapped in Figure 2. Before mechanical stimulation, most epithelial cells displayed a uniform resting $[Ca^{2+}]_i$ of 50–100 nM, although some cells had higher levels of up to 160 nM. Immediately after mechanical stimulation, $[Ca^{2+}]_i$ increased at the point of contact and a wave of increasing Ca^{2+} spread throughout the cell in all directions until it reached the outer edges of the cell (Figure 2). After a short delay of ~0.5–1.5 s, waves of increasing Ca^{2+} were then observed in the adjacent cells. These secondary Ca^{2+} waves emanated from the edge of the cell closest to the stimulated cell at the site of the intercellular boundaries and spread to the opposite end of the cell. After a further delay, also of ~0.5–1.5 s, the more distal neighboring cells displayed a similar series of Ca^{2+} waves that spread from one end of the cell to the other. In this cell-by-cell manner, increases in $[Ca^{2+}]_i$ spread to most of the cells in the field of view (Figure 2). These responses have been observed ≥50 times.

The velocity at which these waves of increasing Ca^{2+} spread through and between cells was determined from the times at which a change in fluorescence first appeared within a cell and the time at which the fluorescence change reached the opposite side of the cell. The results of this analysis are shown in Figure 3. The Ca^{2+} wave traveled through the cell cytoplasm at ~25 μm/s; this wave velocity was independent

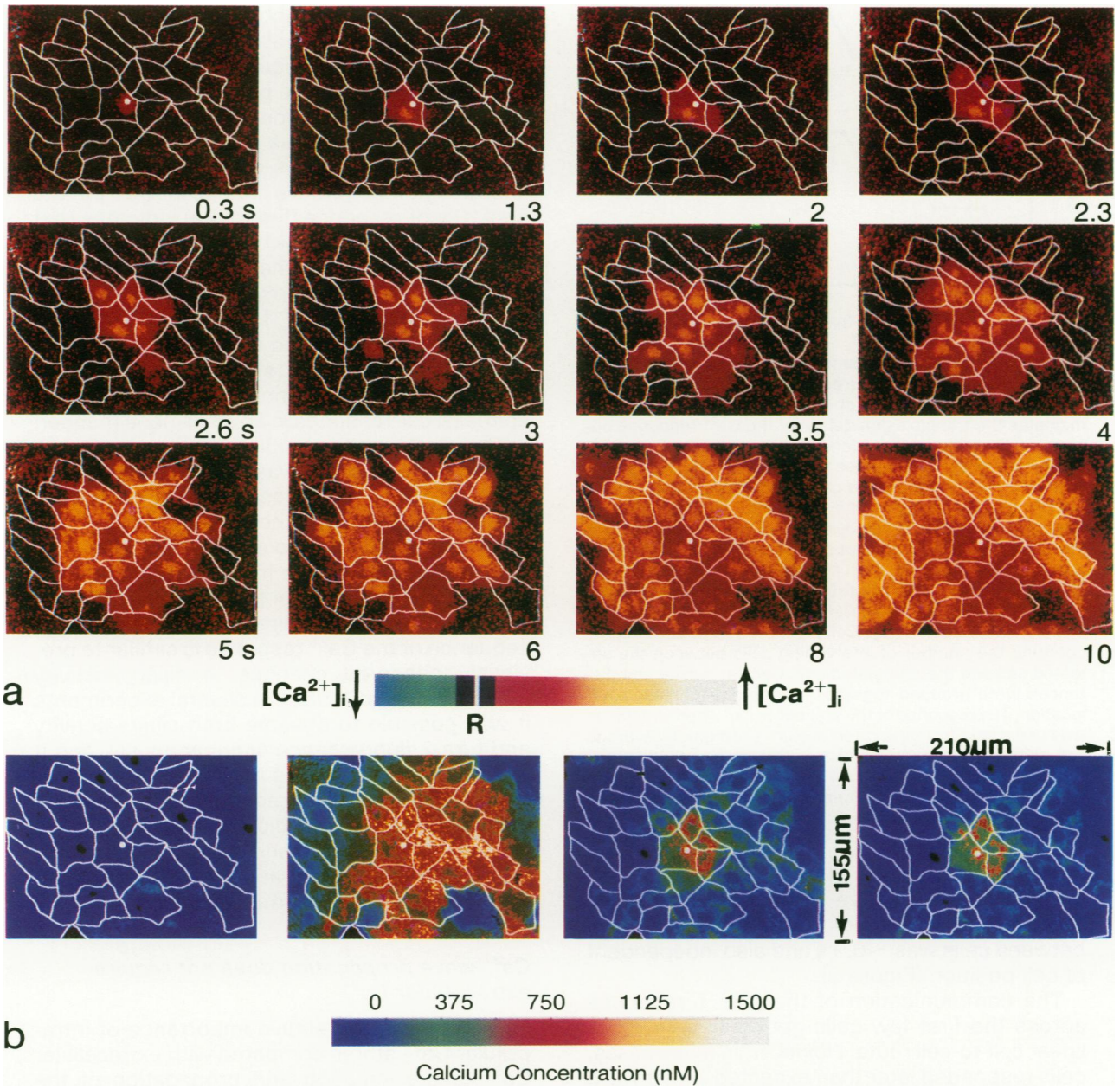

Figure 2. **The effect of mechanical stimulation on $[Ca^{2+}]_i$ of respiratory tract cells in culture, in the presence of extracellular Ca^{2+}.** The white dot indicates the point of mechanical stimulation. The cell boundaries, determined from phase-contrast and fluorescence images, are outlined in white. (a) The relative changes in $[Ca^{2+}]_i$ (indicated by the upper scale bar) with respect to time after stimulation (seconds, indicated below each panel) determined at a single wavelength (380 nm). Increases in $[Ca^{2+}]_i$ (indicated by red/orange) spread out from the point of stimulation to encompass almost all the cells in the field of view. The initial or resting $[Ca^{2+}]_i$ (R) is highlighted for clarity (in scale bar only) by the white line. The black zone on either side of this line represents a threshold set to reduce noise. Increases or decreases in $[Ca^{2+}]_i$ that are within this threshold region remain black. The effect of mechanical stimulation on these cells was also examined, with 340 nm light acting as the predominant excitation wavelength. Under these conditions, the spatial patterns of changing fluorescence were almost identical to those observed at 380 nm, except that fluorescence changes were inverted. (b) Absolute $[Ca^{2+}]_i$ maps were constructed by ratio imaging at wider time intervals. Actual $[Ca^{2+}]_i$ levels are indicated by the lower calibration bar. Vertical and horizontal dimension scale bars are different because of the aspect-ratio of the camera.

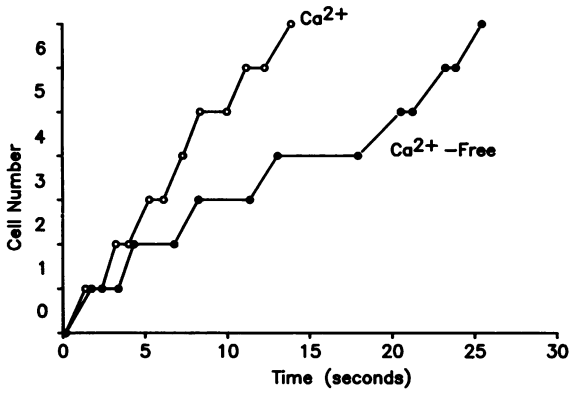

***Figure 3.* A graphic representation of the transmission of the Ca^{2+} wave across cultured epithelial cells in the presence (○) and absence (●) of extracellular Ca^{2+}.** Each plot indicates the transmission delays of the Ca^{2+} response between cells (horizontal sections) and the velocity of the Ca^{2+} wave (slopes) as it propagates through each cell. Data points plotted are the mean values of the time at which increases in Ca^{2+} were first observed in cells and the time taken for a response to reach the opposite side of the cell. Cell borders are represented by tick marks on vertical axis. Each value was calculated from 4 to 40 cells (total of 163), from three cultures obtained from two rabbits. Standard deviations are not shown because they interfere with the representation of the transmission delay. Cell position was determined by counting the number of intervening cells between the selected cell and the stimulated cell. Cells at similar cell distances were grouped together irrespective of their polar location. The response of the first cell in Ca^{2+}-free conditions was estimated from the rates of $[Ca^{2+}]_i$ reduction. Average cell diameter in Ca^{2+} was 28.3 ± 9.3 μm, n = 80; in Ca^{2+}-free was 30.6 ± 11.3 μm, n = 73. Cells were measured in vertical and horizontal directions to take into account the aspect-ratio of the images.

of cell position relative to the stimulated cell. The average duration of the transmission delay between cells was ~0.9 s and also independent of cell position (Figure 3).

The communication of the $[Ca^{2+}]_i$ response across the first few cells generally followed a linear cell-to-cell route. However, in many cases, cells responded later than expected with a wave of increasing $[Ca^{2+}]_i$ beginning from a distal site adjacent to another responding cell. The routes of communication of the Ca^{2+} response became less distinct as both the distance from the stimulated cell and the numbers of responding cells increased.

After 10–12 s, most of the cells within the field of view (4–6 in all directions) had responded by increasing $[Ca^{2+}]_i$ (Figure 2). In some cases, propagation of the response ceased after two or three cells in a few directions. Twenty seconds after stimulation $[Ca^{2+}]_i$ had risen to between 800 and 1200 nM in the stimulated cell and its immediate neighbors (Figure 2b). However, by comparing the absolute (Figure 2b) and relative $[Ca^{2+}]_i$ maps (Figure 2a), it becomes apparent that many of the peripheral cells had already reached a maximum $[Ca^{2+}]_i$ within 10 s and were in the process of restoring $[Ca^{2+}]_i$ to their resting levels 20 s after stimulation. The uniformity of the relative change in $[Ca^{2+}]_i$ in the first 10 s of response (Figure 2a) indicates that all the cells responded with an increase in $[Ca^{2+}]_i$ to at least 800 nM. When the propagation of the $[Ca^{2+}]_i$ wave was terminated, the last cell to respond achieved $[Ca^{2+}]_i$ increases that were similar to those reached in preceding cells but the next adjacent cell showed no change in $[Ca^{2+}]_i$. These observations suggest that each epithelial cell responds in an all-or-none manner.

The $[Ca^{2+}]_i$ in the stimulated cell remained elevated for ≥45 s and was often the last cell to recover. Distal cells frequently restored their $[Ca^{2+}]_i$ to resting levels more quickly. As a result, the increasing Ca^{2+} response spread concentrically across the culture from the point of stimulation, whereas normal $[Ca^{2+}]_i$ levels were restored in the opposite direction (Figure 2b). This sequence of the Ca^{2+} response is similar to previously observed changes in ciliary activity (Sanderson *et al.*, 1988). In several experiments it was possible to observe both ciliary activity and fura-2 fluorescence simultaneously, and it was clear that increases in $[Ca^{2+}]_i$ preceded increases in ciliary beat frequency. The presence of fura-2 in these cells did not influence the ciliary response lag, rise, and recovery times (data not shown), demonstrating that intracellular fura-2 did not affect normal cellular activity.

Ca^{2+} wave propagation does not require extracellular Ca^{2+}

To determine the relative importance of intracellular Ca^{2+} stores compared with extracellular Ca^{2+} in the initiation and propagation of the changes in $[Ca^{2+}]_i$, cultured cells were mechanically stimulated in the absence of extracellular Ca^{2+}. Under these conditions the stimulated cell did not display an increase in $[Ca^{2+}]_i$ but instead displayed either no change or a decrease in $[Ca^{2+}]_i$ (Figure 4a, n = 10). The ciliary beat frequency of the stimulated cell, observed at the end of two experiments, was also reduced under these conditions. These results are in agreement with our earlier findings that mechanical stimulation in the absence of extracellular Ca^{2+} reduced ciliary beat frequency (Sanderson and Dirksen, 1986). A decrease in $[Ca^{2+}]_i$ was par-

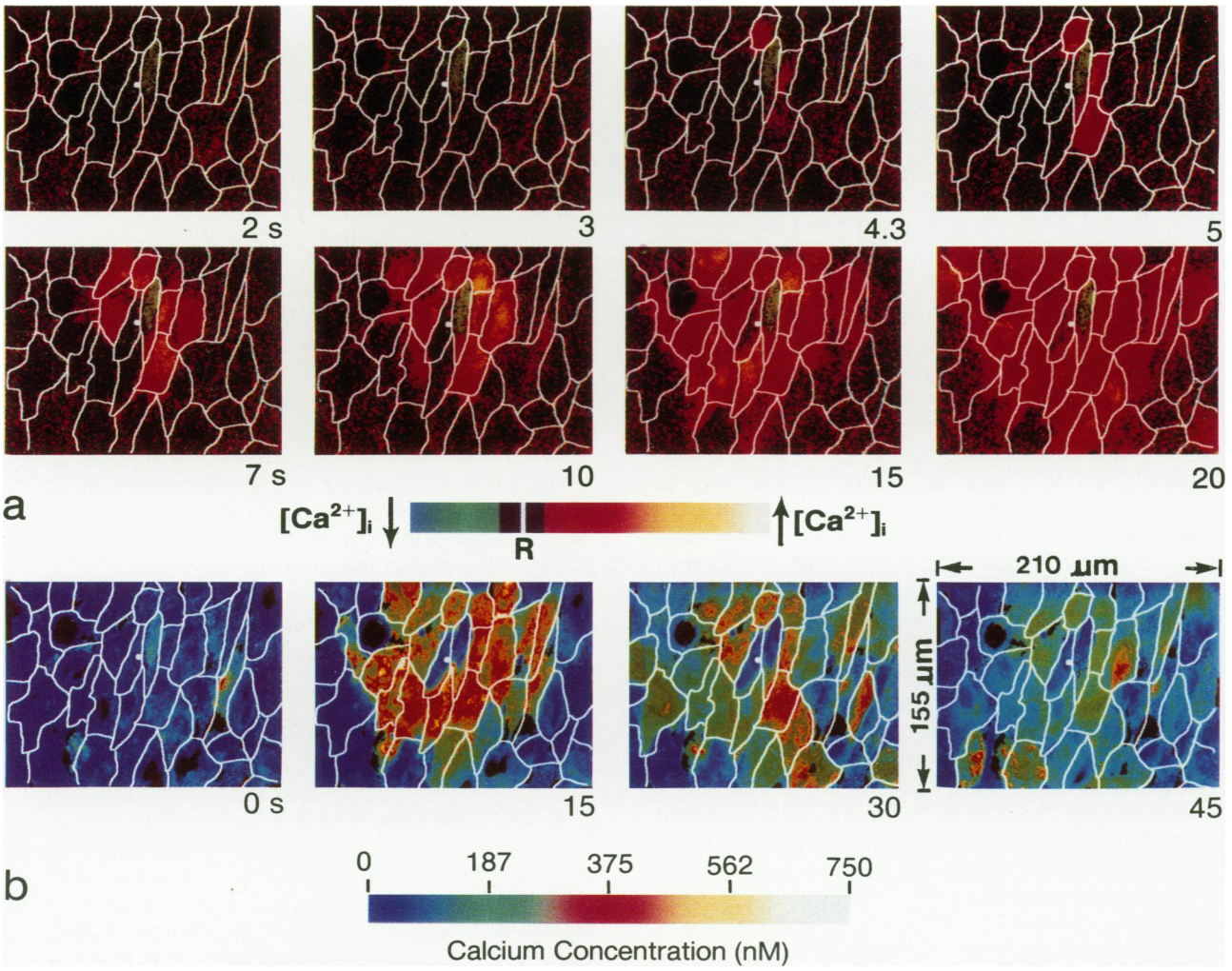

Figure 4. **The effect of mechanical stimulation on $[Ca^{2+}]_i$ of respiratory tract cells in culture in the absence of extracellular Ca^{2+}.** See Figure 2 for panel and scale bar details. (a) Relative changes in $[Ca^{2+}]_i$ (indicated by the upper scale bar) with respect to time after stimulation (seconds, indicated below each panel) were determined at a single wavelength (380 nm). The stimulated cell and one immediate neighbor show either no change (black) or a decrease (green) in $[Ca^{2+}]_i$. It is likely—because mechanical stimulation was close to the cell boundary—that the adjacent cell was also mechanically stimulated. A decrease in $[Ca^{2+}]_i$ is observed in the cell that had a higher initial $[Ca^{2+}]_i$ level (Figure 4b). The adjacent nonstimulated cells show a distinct increase (red) in $[Ca^{2+}]_i$. (b) Absolute $[Ca^{2+}]_i$ maps constructed by ratio imaging at wider time intervals confirm the low $[Ca^{2+}]_i$ in the stimulated cell. Actual $[Ca^{2+}]_i$ levels are indicated by the lower calibration bar. The maximum increase in $[Ca^{2+}]_i$ reached in these cells under Ca^{2+}-free conditions is less than that shown for cells in the presence of extracellular Ca^{2+}. However, in other experiments $[Ca^{2+}]_i$ maximum concentrations of ~1 µM were attained in cells in Ca^{2+}-free conditions.

ticularly evident in cells with initial resting $[Ca^{2+}]_i$ values slightly higher than those of the surrounding cells. After stimulation the $[Ca^{2+}]_i$ in these cells decreased from 150 nM to 50 nM. In spite of this, it was particularly interesting to observe that many of the adjacent cells still displayed an increase in $[Ca^{2+}]_i$ (Figures 3 and 4). However, the rate and pattern of changes in $[Ca^{2+}]_i$ were different from those observed in the presence of extracellular Ca^{2+}. Instead of the rapid concentric spreading of increased $[Ca^{2+}]_i$ across contiguous cells, in the absence of extracellular Ca^{2+}, cells responded in a slower and less organized manner, giving rise to a patchwork pattern of cells with increased $[Ca^{2+}]_i$. A number of cells also displayed increases in $[Ca^{2+}]_i$ that began within a cell at sites distant from other responding cells. This result rarely occurred in cells in the presence of extracellular Ca^{2+}. The velocity of the Ca^{2+} wave in the cytoplasm was reduced to ~19 µm/s, and the transmission delay between cells was in-

creased to ~2.2 s under Ca^{2+}-free conditions (Figures 3 and 4). In combination, these changes resulted in a slowed overall propagation of the response to more distal cells. One consequence of this was that cells near the stimulated cell restored their $[Ca^{2+}]_i$ to resting values before more distal cells had responded. Interestingly, our present data suggest that neither the magnitude of the increase in $[Ca^{2+}]_i$ nor the cell distances over which the response traveled appeared to be affected by the absence of extracellular Ca^{2+}. In previous studies we reported that adjacent cells did not appear to increase their ciliary beat frequency after mechanical stimulation in the absence of extracellular Ca^{2+} (Sanderson and Dirksen, 1986). However, we have reexamined the adjacent cell response and found that 9/9 adjacent cells showed an increase in beat frequency under Ca^{2+}-free conditions. The lag time of this response was slower (4.8 ± 2.11 s, mean ± SD) than that of adjacent cells in the presence of Ca^{2+} (1.5 ± 0.4 s, mean ± SD), but the magnitude of the frequency increase was similar. These results are consistent with the observed changes in $[Ca^{2+}]_i$ under Ca^{2+}-free conditions. One possible explanation for apparent differences between these and previous results is that prolonged exposure to Ca^{2+}-free solutions may deplete the cell of intracellular Ca^{2+}, reducing the ability of the cell to respond. The cells responding at distal sites or those that were bypassed may be examples of this altered response.

Effect of microinjection of IP_3

The ability of the cells to respond to mechanical stimulation in the absence of extracellular Ca^{2+} suggests that elevations in $[Ca^{2+}]_i$ are brought about by the release of Ca^{2+} from intracellular stores. Because inositol 1,4,5-trisphosphate (IP_3) has been identified as a major intracellular messenger that is responsible for the release of Ca^{2+} from intracellular stores in a variety of cells (Berridge and Irvine, 1989), we iontophoretically injected IP_3 into respiratory tract epithelial cells in the absence of extracellular Ca^{2+} (Sanderson *et al.,* 1989). The cells responded to penetration with the micropipette in a manner typical of mechanical stimulation in the absence of extracellular Ca^{2+}; the penetrated cell decreased its $[Ca^{2+}]_i$ and adjacent cells increased their $[Ca^{2+}]_i$. Before IP_3 was iontophoretically injected, a postpenetration recovery period of ~1 min was allowed. After IP_3 was injected (150-ms pulse) into the cell, a transient wave of changing fluorescence, indicating an increase in $[Ca^{2+}]_i$, was observed spreading from the micropipette tip to the borders of the cell (Figure 5, n = 30). The Ca^{2+} wave spread at an average rate of 36 μm/s (n = 3). The Ca^{2+} response was communicated to the adjacent cells and was initially observed in the first, second, and third adjacent cells after an average time of 4.27 (n = 10), 7.16 (n = 8), and 10.12 (n = 5) s, respectively. The Ca^{2+} wave propagated across the first, second, and third adjacent cells in 1.55, 1.45, and 1.48 s, respectively. These rates of communication are very similar to those observed in response to mechanical stimulation under Ca^{2+}-free conditions (Figure 3). In control experiments, in which saline was injected (n = 8), an increase in $[Ca^{2+}]_i$ was not observed. Iontophoretic injection was used in preference to pressure injection to avoid the possibility of mechanical stimulation during injection. The failure of a saline injection to initiate a $[Ca^{2+}]_i$ wave indicated that iontophoretic injection itself did not produce mechanical stimulation.

Effect of halothane on the mechanosensitive response

To investigate the possibility that communication of the Ca^{2+}-response occurs through gap junctions, we have examined the effects of 2-bromo-2-chloro-1,1,1-trifluoroethane (halothane) on mechanosensitivity. The anesthetic halothane has been shown to uncouple cells by blocking gap junctions (Burt and Spray, 1989). Before the addition of halothane, the mechanosensitivity of the cells was confirmed. Fourteen cells from three cultures were stimulated under control conditions in Hanks' balanced salt solution (HBSS), and all responded by producing an increase in $[Ca^{2+}]_i$ in both the stimulated and adjacent cells as has been described. Exposure of the cells to halothane initially induced an increase in $[Ca^{2+}]_i$ and ciliary beat frequency, but both of these subsequently decreased with time. In the presence of 4–5 mM halothane, mechanical stimulation again induced an increase in $[Ca^{2+}]_i$ that spread out from the point of contact to the boundaries of the stimulated cell (Figure 6). However, in contrast to the control response, in most cases (11/15) no communication of the $[Ca^{2+}]_i$ wave to the adjacent cell occurred; weak communication compared with the control experiments occurred in three cases. When the halothane was removed from the culture by washing with HBSS, a communicated Ca^{2+} response was again observed in

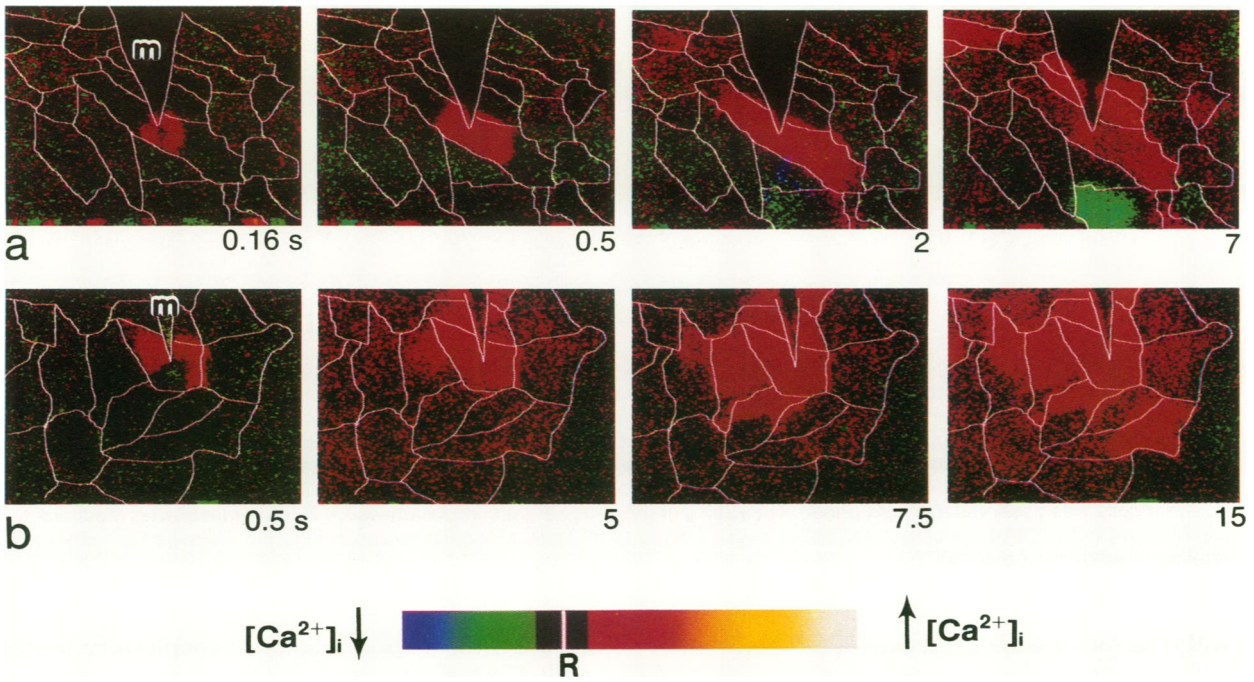

Figure 5. **Two examples of the effect of iontophoretic injection of IP_3 into cultured epithelial cells in the absence of extracellular Ca^{2+}.** The cell borders and position of the injection micropipette (m) are highlighted by the white lines. The time each image was obtained after injection is indicated below each panel (seconds). (a) In the upper sequence of 4, an injection of IP_3 (duration = 150 ms) produced a wave of increasing $[Ca^{2+}]_i$ that originated from the pipette tip and spread along the cell, resulting in the elevation of $[Ca^{2+}]_i$ throughout the cell. The velocity of the spreading Ca^{2+} wave in this cell was measured to be 32.7 μm/s. Subsequently, adjacent cells displayed an increase in $[Ca^{2+}]_i$ with a time course similar to that induced by mechanical stimulation in Ca^{2+}-free conditions. (b) This second example of intracellular injection of IP_3, sampled at greater time intervals, clearly demonstrates the communication of an elevation in $[Ca^{2+}]_i$ between adjacent cells. Relative changes in $[Ca^{2+}]_i$ are indicated by color bar. Magnification as in Figures 2 and 4.

11/14 cells that were tested within 1 h. When halothane was used at 3 mM, a restricted Ca^{2+} response was only observed in 4/10 trials. A similar restriction of the Ca^{2+} wave to the stimulated cell was observed in the presence of a 3 mM octanol in 1% dimethylsulfoxide (DMSO) and HBSS.

Discussion

The results presented in this paper clearly show that mechanical stimulation of respiratory tract epithelial cells induces an increase in $[Ca^{2+}]_i$ that is propagated by intercellular communication. This response to mechanical stimulation can be divided into two stages, the response of the stimulated cell and the response of neighboring cells. The increase in $[Ca^{2+}]_i$ in the stimulated cell is dependent on extracellular Ca^{2+}. This finding could be explained by the opening of stretch-activated channels in response to mechanical stimulation; Ca^{2+}-conducting, stretch-activated channels have been identified in a variety of cell types by patch-clamp techniques (Guharay and Sachs, 1984; Christensen, 1987; Lansman *et al.*, 1987; Gustin *et al.*, 1988; Sachs, 1988). In the absence of extracellular Ca^{2+}, an opening of apical membrane channels permeable to Ca^{2+} could allow an efflux of Ca^{2+} from the stimulated cell and account for the observed decrease in $[Ca^{2+}]_i$. This decrease in $[Ca^{2+}]_i$ implies that Ca^{2+} would not be available to move from the stimulated cell into neighboring cells. The important conclusion drawn from these results is that, although Ca^{2+} is involved in these responses, Ca^{2+} itself is not required to transduce mechanical stimulation or initiate intercellular communication.

In contrast to the stimulated cell, the increase in $[Ca^{2+}]_i$ in neighboring cells is not dependent on extracellular Ca^{2+} or on an increase in $[Ca^{2+}]_i$ in the stimulated cell. However, the injection of IP_3 into one cell resulted in the initiation and propagation of a Ca^{2+} wave in the injected and neighboring cells. These results are consistent

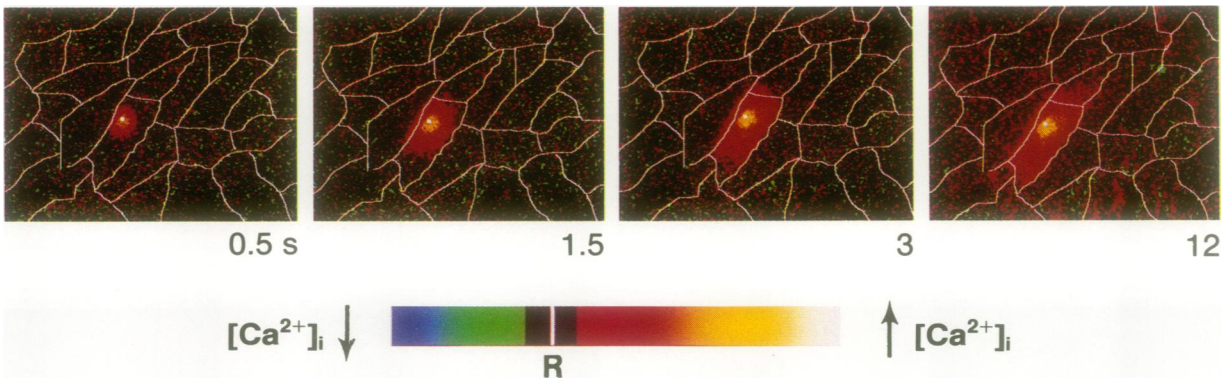

Figure 6. **The effect of halothane (5 mM) on the response of the cultured epithelial cells to mechanical stimulation in the presence of extracellular Ca^{2+}.** Cell borders and point of stimulation are outlined in white; time from stimulation, in seconds, is shown below each panel. Mechanical stimulation induced a wave of increasing $[Ca^{2+}]_i$ that spread throughout the cell from the point of stimulation (3 left panels). The time course and magnitude of the response in the stimulated cell was similar to that in cells stimulated in the absence of halothane. However, no communication of the Ca^{2+} response to adjacent cells was observed (far right panel). The $[Ca^{2+}]_i$ of the cells before stimulation was ~70 nM or less; after mechanical stimulation the $[Ca^{2+}]_i$ of the stimulated cell increased by up to 800 nM. Magnification as in Figure 2. Relative Ca^{2+} changes are indicated by the color bar.

with the fact that IP_3 releases Ca^{2+} from intracellular stores (Berridge and Irvine, 1989) and support our hypothesis that IP_3 is elevated by mechanical stimulation and acts as a messenger in cell-to-cell communication in epithelial cells. We cannot determine from our data if the injected IP_3 itself spreads throughout the culture by diffusion or if a regenerative production of IP_3, as suggested for other cell types (Eberhard and Holz, 1988; Berridge and Irvine, 1989; O'Sullivan *et al.*, 1989; Whitaker, 1989), is required to propagate the intracellular Ca^{2+} wave.

An elevation of IP_3 would be expected to increase intracellular Ca^{2+} in the stimulated cell even in the absence of extracellular Ca^{2+}. However, the observation that the stimulated cell displayed either no change or a reduction in intracellular Ca^{2+} would be explained if membrane channels were opened, as suggested, by mechanical stimulation; any Ca^{2+} released by IP_3 could not accumulate within the cell. The mechanism by which IP_3 could be produced in the mechanically stimulated cell is unknown, but enzyme (phosphoinositidase) activation brought about by conformational changes induced by means other than receptor-ligand interactions has been previously suggested (Vergara *et al.*, 1985; Sachs, 1988).

Because the microinjection of IP_3 mimics the mechanosensitive response and an elevation of $[Ca^{2+}]_i$ is not required in the stimulated cell, we favor the suggestion that IP_3 mediates cellular communication by passing to adjacent cells through the gap junctions of respiratory tract cells (Sanderson *et al.*, 1989). A similar mechanism has been postulated for rat hepatocytes (Sáez *et al.*, 1989) and cultured kidney cells (Atkinson, 1989). The simplest mechanism of communication is the diffusion of the messenger through the cell cytoplasm and gap junctions to reach adjacent cells. However, the cell distances over which these $[Ca^{2+}]_i$ waves travel and the all-or-none response of each cell would be more easily explained by a regenerative process in adjacent cells. The activation of phospholipase C by Ca^{2+} has been proposed as a mechanism for the regeneration of IP_3 (Eberhard and Holz, 1988; Whitaker, 1989). Ca^{2+}-induced Ca^{2+} release (Fabiato and Fabiato, 1977; Endo, 1985) is another mechanism by which a regenerative wave of Ca^{2+} could be produced, and, although this mechanism does not appear to be essential in the stimulated cell under Ca^{2+}-free conditions, we cannot discount the possibility that this process occurs in adjacent cells. Ca^{2+} may also pass from one cell to the next and facilitate the effects of IP_3 (Sáez *et al.*, 1989).

In other types of ciliated cells, transduction of a mechanical stimulus followed by cell communication occurred quickly and was associated with membrane depolarization; a ciliary arrest response was observed within 100 ms in cells that were 10 cells away from the point of stimulation (Murakami and Machemer, 1982; Saimi *et al.*, 1983; Stommel, 1984; Reed and Satir, 1986). In comparison, the propagation of the increase in $[Ca^{2+}]_i$ observed in respiratory

tract cells is slow and, therefore, is probably not directly associated with changes in membrane potential.

The observations that the course of the Ca^{2+} response followed nonlinear pathways throughout the culture suggest that communication between cells occurred at specific locations, such as gap junctions. Because halothane and octanol have been demonstrated to reduce electrical coupling between cells (Johnston *et al.,* 1980; Burt and Spray, 1989) (although this has not, as yet, been specifically demonstrated for respiratory tract cells), the restriction of the Ca^{2+} response to the mechanically stimulated cell in the presence of halothane or octanol is compatible with the suggestion that the communication of the Ca^{2+} response occurs through gap junctions. Halothane is not a specific gap-junction inhibitor, and a similar restricted elevation in $[Ca^{2+}]_i$ might also result if halothane interfered with the mechanism that transduced mechanical stimulation. However, this does not seem to be the case, because the initial spread of increasing $[Ca^{2+}]_i$ throughout the stimulated cell in halothane was similar in both magnitude and speed to that observed in cells in HBSS. Furthermore, halothane does not appear to inhibit the production of IP_3 (Bazil and Minneman, 1989) but can inhibit IP_3 phosphatase (Foster *et al.,* 1989). This latter effect may, in fact, contribute to a greater accumulation of IP_3. It has also been suggested that increases in $[Ca^{2+}]_i$ reduce gap-junction conductance, but the occurrence of intercellular communication in these cells supports the idea that normal variations in $[Ca^{2+}]_i$ do not completely inhibit cell-to-cell communication (Spray and Bennet, 1985).

The magnitude of the increase in $[Ca^{2+}]_i$ in neighboring cells appeared to be similar in both the presence and absence of extracellular Ca^{2+}, suggesting that a contribution of extracellular Ca^{2+} to the adjacent cell response is not essential. However, extracellular Ca^{2+} clearly augments the velocity at which these responses spread throughout the culture. This influence of extracellular Ca^{2+} on the response may be indirect; the absence of extracellular Ca^{2+} could act to deplete intracellular stores of Ca^{2+}. Alternatively, extracellular Ca^{2+} may enter the cell (IP_3 and inositol 1,3,4,5,-tetrakisphosphate [IP_4] have been implicated as mediators of external Ca^{2+} influx [Irvine, 1989]) and contribute directly to the Ca^{2+} response by a Ca^{2+}-induced Ca^{2+} release (Fabiato and Fabiato, 1977; Endo, 1985) and/or Ca^{2+}-induced IP_3 regeneration (Eberhard and Holz, 1988; Whitaker, 1989).

In summary, because of the similarity of the intercellular propagating waves of $[Ca^{2+}]_i$ initiated by either mechanical stimulation or iontophoretic injection of IP_3, we suggest that IP_3 may be elevated in a mechanically stimulated cell and that IP_3 mediates signal propagation between respiratory tract cells by releasing intracellular Ca^{2+}. Extracellular Ca^{2+} has more of a facilitating role in these responses. Similar Ca^{2+} waves that propagate between cells have been observed in a variety of cells, including astrocytes (Charles *et al.,* 1990; Cornell-Bell *et al.,* 1990), cultured kidney cells (Atkinson, 1989), and endothelial cells (Jacob, 1990); we suggest that this type of Ca^{2+} response may represent a common mechanism for the integration of cellular activity. Respiratory tract epithelial cells in culture provide an experimental model of cellular systems in which a specific stimulus is transduced and translated into an identifiable multicellular response. The study of signal propagation in respiratory tract cells will not only contribute to our understanding of the control of mucus transport but may also provide valuable information pertaining to intercellular communication and Ca^{2+} signaling in other cell systems.

Methods

Culture of epithelial cells

The procedures for culturing ciliated cells have been published in detail elsewhere (Sanderson and Dirksen, 1985; Sanderson *et al.,* 1988), and these methods will only be briefly reviewed here. The tracheal mucosa was dissected from New Zealand White rabbits and cut into small explants; transferred to round glass cover-slips coated with a collagen gel; covered with a meniscus of nutrient medium consisting of 10% fetal bovine serum, Dulbecco's modified Eagle medium, antibiotics penicillin, and streptomycin (GIBCO Laboratories, Grand Island, NY); and cultured at 37°C in a 10% CO_2 atmosphere. Outgrowths of ciliated cells were evident after 24–36 h; cultures were generally 7–10 d old when used. Collagen gels were prepared with collagen extracted from rat tail tendons with acetic acid.

Measurement of intracellular calcium

Cell cultures were loaded with fura-2 by incubation in 5 μM fura-2-AM (Calbiochem, La Jolla, CA) in HBSS (GIBCO Laboratories) for 1 h at 37°C, washed with fresh HBSS, and left for at least 30 min before use. Cells loaded with fura-2 displayed bright stable fluorescence; unloaded cells possessed virtually no autofluorescence. Fluorescence was observed at room temperature (21–24°C) with a Nikon (Garden City, NY) Diaphot microscope equipped with a ×40, 1.4 N.A. oil-immersion objective and quartz or fused-silica optical elements. Microscope images were viewed with a silicone-intensifier target (SIT) camera (Cohu, San Diego, CA); the automatic gain circuit (AGC) was disabled and placed, with target high voltage and black level, under man-

ual control. Video images were recorded by an optical memory disc recorder, also with the AGC disabled (Panasonic [Secaucus, NJ] TQ2026F). IP_3 images were originally recorded on video tape and transferred to optical disc for analysis. For analysis, images were replayed, digitized, and averaged with the aid of a 512 × 480 pixels × 256 gray levels frame grabber (Data Translation, Marlboro, MA, DT2861), an auxiliary frame processor (Data Translation, DT2858), and an AT-286 microcomputer (American Research, Monterey Park, CA, turbo 12). Images were displayed on an RGB monitor (Sony [Parkridge, NJ] PVM1271Q) and photographed with an Imagecorder (IC 4520 Focus Graphics, Foster City, CA). Dual excitation light wavelengths of 340 nm (half band width: 11 nm) and 380 nm (half band width: 13 nm) were obtained from a 100-W mercury arc by filters (Omega Optical, Brattleboro, VT) in combination with a 405-nm dichroic mirror. Emission spectra were observed through a 510 nm filter (half band pass: 40 nm). Neutral-density filters were used in combination with wavelength filters to approximately equalize the intensity of the excitation light. Filters were placed in an automated filter wheel (Kramer Scientific, Yonkers, NY) and selected by computer. A shutter was used to control illumination.

$[Ca^{2+}]_i$ is not usually determined from fura-2 fluorescence at a single wavelength, but changes in fluorescence at a single wavelength do reflect changes in $[Ca^{2+}]_i$. To obtain improved temporal resolution (1/30 s) and to reduce the effects of the lag characteristics of our SIT video camera (Inoué, 1986), changes in fluorescence intensity were recorded primarily at a single wavelength (380 nm). Conventional image pairs at 340 and 380 nm were also recorded at times when cellular fluorescence was changing more slowly, i.e., before mechanical stimulation and after the peak response, to quantitate $[Ca^{2+}]_i$ by ratio imaging and to act as calibration reference points for the single-wavelength recordings. Images were collected in a typical experiment as follows: *1*) before stimulation, 30 images were recorded at 340 and 380 nm. *2*) Continuous recording at 380 nm was then initiated. *3*) After 1 s the mechanical stimulus was applied. *4*) At 20, 40, and 60 s (Figure 2) or 15, 30, and 45 s (Figure 4), the 380-nm filter was switched to 340 nm and 30 frames were recorded before the return to 380 nm. Maps of relative changes in $[Ca^{2+}]_i$ (Figures 2a and 4a) were constructed from these continuous video recordings. For each time point, a ratio image between the initial 380-nm image (obtained before mechanical stimulation $[I_0]$) and a subsequent 380-nm image (obtained at sequential times after stimulation [e.g., I_{t1}, I_{t2}]) was calculated (i.e., I_0/I_{t1}). In contrast, maps of absolute $[Ca^{2+}]_i$ (Figures 2b and 4b) were constructed by substituting the 340/380 image ratio (R) in a pixel-by-pixel basis into the equation $Kd(F_0/F_s)[(R-Rmin)/(Rmax-R)]$ where Kd is the dissociation constant of fura-2 (taken to be 224 nM; see Grynkiewicz *et al.*, 1985), Rmax is the ratio at saturating levels of Ca^{2+}, Rmin is the ratio at 0 Ca^{2+}, and (F_0/F_s) is the ratio at 380 nm of 0 and saturating concentrations of Ca^{2+}. All images were corrected, after background subtraction, with an intensity transform function and a shading map (Walter and Berns, 1986; Williams *et al.*, 1985).

Fluorescence of fura-2 in tracheal ciliated epithelial cells did not match that exhibited by in vitro solutions of fura-2. This observation—that the fluorescence properties of fura-2 are significantly affected by the cytoplasmic environment—has been reported by several other laboratories (Williams *et al.*, 1985, 1987; Scanlon *et al.*, 1987; Wier *et al.*, 1987). Substitution for Rmax, Rmin, and (F_0/F_s) with values obtained from in vitro calibrations into the above equation results in a measured $[Ca^{2+}]_i$ that is up to 50% less than that measured when using in vivo values. As a result, the in vivo calibration values have been used to calculate free $[Ca^{2+}]_i$.

In vivo calibration. Cells were loaded with fura-2-AM, washed with Ca^{2+}-free saline solution containing 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetra-acetic acid (EGTA), 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 103 mM NaCl, 1 mM free MgCl, and 5 mM KCl at pH 7.4 and exposed to 5 μM ionomycin (Calbiochem) in Ca^{2+}-free saline solution. After 3–5 min, when no further changes in fura-2 fluorescence were observed, cellular fluorescence was recorded. The bathing solution was exchanged for saline containing 1 mM Ca^{2+} and 5 μM ionomycin and the cells were left to equilibrate for 5 min before recording cellular fluorescence. The in vivo Rmax, Rmin, and (F_0/F_s) were determined to be 5.52, 0.36, and 5.02, respectively.

In vitro calibration. Three microliters of calibration solution were placed between two cover glasses and the resulting fluorescence recorded. Calibration solutions consisted of 50 μM fura-2 salt in 100 mM KCl, 25 mM HEPES, and 1 mM free Mg^{2+} at pH 7.1 or 7.4 with a free Ca^{2+} concentration adjusted between 0 or 1 mM using 10 mM EGTA as a buffer. In vitro values for Rmax, Rmin and (F_0/F_s) were determined to be 10.5, 0.25, and 8.5, respectively.

Nonlinearities in camera gain and illumination were corrected by the application of a linear-intensity transform function and shading map. Each image was divided up into 16 × 16 pixel units. The linear-intensity transform function was constructed, after background subtraction, from the average digital gray level in each area when a uniform light source of varying intensities (obtained by neutral density filters) was imaged by the system. A shading map for 340- and 380-nm light was obtained by examining a bright field of fura-2 fluorescence. The intensity-transform function was applied in the form of a memory-resident look-up table after the background had been subtracted. The resulting image was corrected for shading by dividing it, on a pixel-by-pixel basis, by the shading map and multiplying the result by the mean gray level of the map. After correction and the necessary ratio processing, each image was scaled for pseudocolored display and photographic recording. Many of these practical and technical aspects of fluorescence imaging have been described in a collection of papers in a special edition of Cell Calcium (1990, *11*, 55–249).

Mechanical stimulation of cells

Single ciliated cells within the cultured epithelium were mechanically stimulated in HBSS by distorting their apical membrane with a fine glass microprobe (tip diam ~ 1 μm; effective tip displacement, 2–5 μm) driven by a piezoelectric device (Sanderson and Dirksen, 1986). HBSS contained 5.3 mM KCl, 1.2 mM $CaCl_2$, 25 mM HEPES, 5.5 mM D-glucose, 136 mM NaCl, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 0.3 mM KH_2PO_4, and 0.3 mM Na_2HPO_4. For Ca^{2+}-free conditions the cells were washed with several exchanges of Ca^{2+}-free saline consisting of 10 mM EGTA, 25 mM HEPES, 103 mM NaCl, 1 mM free MgCl, 5 mM KCl, and 5.5 mM D-glucose at pH 7.4. NaCl concentration was adjusted to compensate for the removal of Ca^{2+}. The exchange of HBSS for Ca^{2+}-free saline did not appear to affect the cell. Long exposure (≥20 min) to Ca^{2+}-free solution resulted in changes in cell shape.

Exposure of cells to halothane

A saturated solution of halothane (Sigma Chemical, St. Louis, MO) in HBSS was made by mixing 30 ml HBSS with

10 ml halothane for ≥3 h at room temperature. Solutions were also stored overnight at 4°C. Similar saturated solutions of halothane have been measured by chromatography techniques to contain 16–17 mM halothane (Burt and Spray, 1989). In our study we have assumed a concentration of 16 mM for our saturated halothane solution. Experimental solutions were made by diluting the saturated stock solution with HBSS in capped tubes.

Iontophoretic injection

Iontophoretic injections were performed with the use of noncommercial equipment; a power operational amplifier (PA83, Apex Microtechnology, Tucson, AZ) was used to generate the injecting current. Micropipettes were filled with a control solution containing 100 mM KCl, 10 mM HEPES (pH 7.1–7.4), and 0.1% 6-carboxyfluorescein (Calbiochem); IP_3 (Calbiochem) was added to a final concentration of 1.6 mM when required. Carboxyfluorescein was included for visual confirmation of injection. An injection pulse of ~40–60 nA for 150 ms was used; a backing current of 1–3 nA was applied during impalement and between injections.

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References

Atkinson, M.M. (1989). Calcium and inositol 1,4,5-trisphosphate traverse gap junctional channels. J. Cell Biol. *109,* 100a.

Bazil, C.W., and Minneman, K.P. (1989). Effects of clinically effective concentrations of halothane on adrenergic and cholinergic synapses in rat brain in vitro. J. Pharmacol. Exp. Ther. *248,* 143–148.

Berridge, M.J., and Irvine, R.F. (1989). Inositol phosphates and cell signalling. Nature, *341,* 197–205.

Burt, J.M., and Spray, D.C. (1989). Volatile anesthetics block intercellular communication between neonatal rat myocardial cells. Circ. Res. *65,* 829–837.

Caveney, S. (1985). Intercellular communication. In: Comprehensive Insect Physiology, Biochemistry and Pharmacology, ed. G.A. Kerkut and L.I. Gilbert, Oxford, UK: Pergamon Press, 319–370.

Charles, A., Merrill, J., Dirksen, E., and Sanderson, M. (1990). Elevation and oscillation of $[Ca^{2+}]_i$ and intercellular communication in glial cells in response to trauma and glutamate. Soc. Neurosci. Abstr. (in press).

Christensen, O. (1987). Mediation of cell volume regulation by Ca^{2+} influx through stretch-activated channels. Nature *330,* 66–68.

Cornell-Bell, A.H., Finkbeiner, S.M., Cooper, M.S., and Smith, S.J. (1990). Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. Science, *247,* 470–473.

Eberhard, D.A., and Holz, R.W. (1988). Intracellular Ca^{2+} activates phospholipase C. Trends Neurosci. *11,* 517–520.

Endo, M. (1985). Calcium release from sarcoplasmic reticulum. Curr. Top. Membr. Transp. *25,* 181–230.

Fabiato, A., and Fabiato, F. (1977). Calcium release from the sarcoplasmic reticulum. Circ. Res. *40,* 119–129.

Foster, P.S., Gesini, E., Claudianos, C., Hopkinson, K.C., and Denborough, M.A. (1989). Inositol 1,4,5-trisphosphate phosphatase deficiency and malignant hyperpyrexia in swine. Lancet, *ii,* 124–127.

Fraser, S.E., Green, C.R., Bode, H.R., and Gilula, N.B. (1987). Selective disruption of gap junctional communication interferes with a patterning process in hydra. Science *237,* 49–55.

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

Guharay, F., and Sachs, F. (1984). Stretch-activated single ion channel currents in tissue-cultured embryonic chick skeletal muscle. J. Physiol. *352,* 685–701.

Gustin, M.C., Zhou, X-L., Martinac, B., and Kung, C. (1988). A mechanosensitive ion channel in the yeast plasma membrane. Science *242,* 762–765.

Inoué, S. (1986). Video Microscopy, New York: Plenum Press.

Irvine, R.F. (1989). Functions of inositol phosphates. In: Inositol Lipids in Cell Signalling, ed. R.H. Michell, A.H. Drummond, and C.P. Downes, London: Academic Press, 135–161.

Jacob, R. (1990). Imaging cytoplasmic free calcium in histamine stimulated endothelial cells and in fMet-Leu-Phe stimulated neutrophils. Cell Calcium *11,* 241–249.

Johnston, M.F., Simon, S.A., and Ramon, F. (1980). Interaction of anaesthetics with electrical synapses. Nature *286,* 498–500.

Lansman, J.B., Hallam, T.J., and Rink, T.J. (1987). Single stretch-activated ion channels in vascular endothelial cells as mechanotransducers? Nature *325,* 811–813.

Loewenstein, W.R. (1981). Junctional intercellular communication: the cell-to-cell membrane channel. Physiol. Rev. *61,* 829–913.

Murakami, A., and Machemer, H. (1982). Mechanoreception and signal transmission in the lateral ciliated cells on the gill of *Mytilus.* J. Comp. Physiol. *145,* 351–362.

O'Sullivan, A.J., Cheek, T.R., Moreton, R.B., Berridge M.J., and Burgoyne, R.D. (1989). Localization and heterogeneity of agonist-induced changes in cytosolic calcium concentration in single bovine adrenal chromaffin cells from video imaging of fura-2. EMBO J. *8,* 401–411.

Reed, W., and Satir, P. (1986). Spreading ciliary arrest in a mussel gill epithelium: characterization by quick fixation. J. Cell. Physiol. *126,* 191–205.

Sachs, F. (1988). Mechanical transduction in biological systems. CRC Crit. Rev. Biomed. Eng. *16,* 141–169.

Sáez, J.C., Connor, J.A., Spray, D.C., and Bennett, M.V.L. (1989). Hepatocyte gap junctions are permeable to the sec-

ond messenger, inositol 1,4,5-trisphosphate, and to calcium ions. Proc. Natl. Acad. Sci. USA *86,* 2708–2712.

Saimi, Y., Murakami, A., and Takahashi, K. (1983). Ciliary and electrical responses to intracellular current injection in the ciliated epithelium of the gill of *Mytilus.* Comp. Biochem. Physiol. A Comp. Physiol. *74,* 507–511.

Sanderson, M.J., and Sleigh, M.A. (1981). Ciliary activity of cultured rabbit tracheal epithelium: beat pattern and metachrony. J. Cell Sci. *47,* 331–347.

Sanderson, M.J., and Dirksen, E.R. (1985). A versatile and quantitative computer-assisted photoelectronic technique used for the analysis of ciliary beat cycles. Cell Motil. *5,* 267–292.

Sanderson, M.J., and Dirksen, E.R. (1986). Mechanosensitivity of cultured ciliated cells from the mammalian respiratory tract: implications for the regulation of mucociliary transport. Proc. Natl. Acad. Sci. USA. *83,* 7302–7306.

Sanderson, M.J., Chow, I., and Dirksen, E.R. (1988). Intercellular communication between ciliated cells in culture. Am. J. Physiol. (Cell Physiol. *23*) *254,* C63–C74.

Sanderson, M.J., and Dirksen, E.R. (1988). Fura-2 fluorescence reveals that intercellular communication elevates intracellular calcium in tracheal ciliated cells. Biophys. J. *53,* 52a.

Sanderson, M.J., Charles, A.C., and Dirksen, E.R. (1989). Inositol trisphosphate mediates intercellular communication between ciliated epithelial cells. J. Cell Biol. *109,* 304a.

Scanlon, M., Williams, D.A., and Fay, F.S. (1987). A Ca^{2+}-insensitive form of fura-2 associated with polymorphonuclear leukocytes. J. Biol. Chem. *262,* 6308–6312.

Spray, D.C., and Bennett, M.V.L. (1985). Physiology and pharmacology of gap junctions. Annu. Rev. Physiol. *47,* 281–303.

Stommel, E.W. (1984). Calcium regenerative potentials in *Mytilus edulis* gill abfrontal ciliated epithelial cells. J. Comp. Physiol. A Sens. Neural. Behav. Physiol. *155,* 445–456.

Vergara, J., Tsien, R.Y., and Delay, M. (1985). Inositol 1,4,5-trisphosphate: a possible chemical link in excitation-contraction coupling in muscle. Proc. Natl. Acad. Sci. USA. *82,* 6352–6356.

Walter, R.J., Jr., and Berns, M.W. (1986). Digital image processing and analysis. In: Video Microscopy, ed. S. Inoué, New York: Plenum Press, 327–392.

Whitaker, M. (1989). Phosphoinositide second messengers in eggs and oocytes. In: Inositol Lipids in Cell Signalling, ed. R.H. Michell, A.H. Drummond, and C.P. Downes. London: Academic Press, 459–483.

Wier, W.G., Cannell, M.B., Berlin J.R., Marban, E., and Lederer, W.J. (1987). Cellular and subcellular heterogeneity of $[Ca^{2+}]_i$ in single heart cells revealed by fura-2. Science *235,* 325–328.

Williams, D.A., Fogarty, K.E., Tsien, R.Y., and Fay, F.S. (1985). Calcium gradients in single smooth muscle cells revealed by the digital imaging microscope using fura-2. Nature *318,* 558–561.

Williams, D.A., Becker, P.L., and Fay, F.S. (1987). Regional changes in calcium underlying contraction of single smooth muscle cells. Science *235,* 1644–1648.