ylase 1A gene (*rbc*S 1A) (*15*). The CTP was modified by addition of the first 23 amino acids (MQVWPPIGKKKFETLSYLPDLTDS) because the efficiency of protein import is increased by addition of the sequences from the mature region of this protein. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. To facilitate removal of the remaining *rbc*S sequences from the ADPGPP protein, we added a second protease cleavage site (GGRVNCMQA) between these 23 amino acids of *rbc*S and the NH_2-terminal Met of *E. coli* ADPGPP. The *glgC16* gene was obtained as a Hinc II fragment from plasmid pLP226 (*8*) and cloned into the pUC8 vector at the Hinc II site. To fuse the modified transit peptide coding region to the translation initiation site of the *glgC16* gene, we introduced an Nco I site at the initiating methionine of the *glgC16* gene. Also, to remove 3′ nucleotide sequences, we added a Sac I site downstream of the termination codon of the *glgC16* gene. Both Nco I and Sac I sites were introduced by polymerase chain reaction (PCR) mutagenesis. The modified *Arabidopsis* CTP plus the *glgC16* gene were cloned into pGEM3zf+ (Promega, Madison, WI), digested with Hind III and Sac I, by ligating the *Arabidopsis* CTP as a Hind III–Nco I fragment and *glgC16* as an Nco I–Sac I fragment. The resulting plasmid (pMON20100) consisted of pGEM3zf+, the modified *Arabidopsis* CTP, and the *glgC16* gene. The CTP-*glgC16* gene in the plasmid was in an orientation suitable for transcription by the SP6 promoter resident in the pGEM3zf+ plasmid.

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Intercellular Propagation of Calcium Waves Mediated by Inositol Trisphosphate

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Two types of calcium (Ca^{2+}) signaling—propagating intercellular Ca^{2+} waves of increasing intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and nonpropagating oscillations in $[Ca^{2+}]_i$—co-exist in a variety of cell types. To investigate this difference in Ca^{2+} signaling, airway epithelial cells were loaded with heparin, an inositol 1,4,5-trisphosphate (IP_3) receptor antagonist, by pulsed, high-frequency electroporation. Heparin inhibited propagation of intercellular Ca^{2+} waves but not oscillations of $[Ca^{2+}]_i$. In heparin-free cells, Ca^{2+} waves propagated through cells displaying $[Ca^{2+}]_i$ oscillations. Depletion of intracellular Ca^{2+} pools with the Ca^{2+}-pump inhibitor thapsigargin also inhibited the propagation of Ca^{2+} waves. These studies demonstrate that the release of Ca^{2+} by IP_3 is necessary for the propagation of intercellular Ca^{2+} waves and suggest that IP_3 moves through gap junctions to communicate intercellular Ca^{2+} waves.

Intercellular communication is essential for the function of multicellular systems, but the nature of the signal or signals that pass between cells through gap junctions is not fully established. Both Ca^{2+} and IP_3 have been proposed as intercellular messengers (*1–3*). Nonexcitable cells often respond to agonists by increasing their $[Ca^{2+}]_i$ in an oscillatory manner (*4*), but these oscillations in $[Ca^{2+}]_i$ occur independently of $[Ca^{2+}]_i$ changes in adjacent cells (*3*). In contrast to Ca^{2+} oscillations, a propagating intercellular wave of increased $[Ca^{2+}]_i$ (a Ca^{2+} wave) can be initiated by mechanical stimulation of a single cell in cultures of airway epithelial (*2*), rat brain glial (*3, 5, 6*), or bovine aortic endothelial (*7*) cells or by treatment of astrocytes with glutamate (*8*). In airway epithelial cells Ca^{2+} waves are blocked by the gap junction inhibitor Ca^{2+} halothane (*2*), and in C6 glioma cells only cells transfected with and expressing the gene for the gap junction protein connexin43 propagate Ca^{2+} waves (*6*). These results indicate that Ca^{2+} waves are propagated through gap junctions (*1–3, 5–8*). A role for IP_3 in the communication of Ca^{2+} waves has been proposed because Ca^{2+} waves are propagated in the absence of extracellular Ca^{2+} (*2, 3, 7*), are propagated when Ca^{2+}-induced Ca^{2+} release is inhibited (*5*), and can be initiated by microinjection of IP_3 (*2*). If IP_3 acts as the intercellular messenger for propagation of Ca^{2+} waves, intracellular heparin, an antagonist of the IP_3 receptor (*9*), should block or attenuate the Ca^{2+} wave (*9*).

Traditional loading techniques, such as microinjection, are not well suited for loading the large numbers of cells required for investigation of this multicellular response. Therefore, we used pulsed high-frequency electroporation (PHFE) (*10, 11*) to load cultured cells with heparin (Fig. 1). Because heparin cannot be detected by fluorescence microscopy, cells were simultaneously loaded with fluorescent Texas red–conjugated dextran (TRD) to identify cells that incorporated heparin (Fig. 1) (*11, 12*). PHFE has a major advantage over current-discharge electroporation in that most cells survive (*10*). After PHFE, and loading cells with fura-2 by incubation in fura-2-pentaacetoxymethyl ester (fura-2-AM) (*12*), more than 90% of the heparin-loaded cells retained fura-2 and heparin-loaded ciliated cells continued to display ciliary activity. These results demonstrate the potential of PHFE for loading cells with molecules that are impermeable to the cell membrane because of their large molecular size or ionic charge.

In an area where all cells were loaded with heparin, mechanical stimulation of a single cell increased $[Ca^{2+}]_i$ in the stimulated cell but did not initiate propagation of a Ca^{2+} wave through multiple adjacent cells, even though the increase in $[Ca^{2+}]_i$ of the stimulated cell ranged from 300 nM to >1 μM (Fig. 2A). In a few of these experiments in the heparin-loaded area, $[Ca^{2+}]_i$ increased in single cells directly adjoining the stimulated cell, but an increase of $[Ca^{2+}]_i$ in more distal cells was not observed. The increase in $[Ca^{2+}]_i$ of the stimulated, heparin-loaded cells and our reports that the $[Ca^{2+}]_i$ of a mechanically

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stimulated cell does not increase in the absence of extracellular Ca^{2+} (*2*, *3*, *7*) suggest that the increase in $[Ca^{2+}]_i$ in the stimulated cells results from an influx of Ca^{2+} across the plasma membrane.

To test if PHFE itself prevented wave propagation, we loaded cells with chondroitin sulfate, a glycosaminoglycan similar to heparin that does not block IP_3-mediated release (9). After mechanical stimulation of a single cell, chondroitin sulfate–loaded cells displayed intercellular Ca^{2+} waves similar to those observed in unloaded cells (Fig. 2B) (*2*, *3*, *5–7*). The range of the increase in $[Ca^{2+}]_i$ of the stimulated cell was similar to that observed in stimulated, heparin-loaded cells. The findings that heparin blocks the propagation of Ca^{2+} waves irrespective of the increase in $[Ca^{2+}]_i$ of the stimulated cell and that Ca^{2+} waves can be propagated without a detectable increase in $[Ca^{2+}]_i$ in the stimulated cell (*2*, *3*, *7*) are not consistent with a mechanism in which

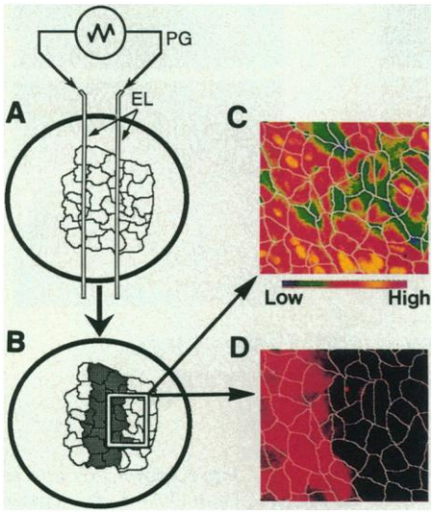

Fig. 1. PHFE of airway epithelial cells. Cells were electroporated in a solution that contained TRD and either heparin or chondroitin sulfate (*11*). A schematic (**A**) showing the electrical field of a pulse generator (PG) applied through two platinum electrodes (EL; 0.025-mm diameter) placed above the cultured cells. After electroporation (**B**), only cells between the electrodes, exposed to the electrical field, became permeable to large molecules. This resulted in a loading border that follows the outline of the cells (shading). An area of cells like that shown schematically in (B) is shown in (C) and (D). Fluorescence image taken at 380 nm [field shown is 155 μm (vertical) by 185 μm (horizontal)]. (**C**) shows that all cells retain fura-2 (scale bar indicates intracellular fura-2 concentration), whereas the TRD fluorescence (**D**) is confined to cells between the electrodes and indicates those cells loaded by electroporation. Images of cells were subjected to background subtraction and contrast enhancement. Cell outlines are drawn in white.

Ca^{2+} alone passes to adjacent cells to propagate the Ca^{2+} wave.

To investigate the nature of the message that is communicated between cells that are not in direct contact with the stimulated cell, we examined the propagation of a Ca^{2+} wave between heparin-free and heparin-loaded cells (Fig. 3). Mechanical stimulation of a cell within the area of heparin-free cells (one to three cells removed from the heparin-loaded cells) initiated an intercellular Ca^{2+} wave that traveled radially away from the stimulated cell. At the boundary between the heparin-free and heparin-loaded cells, the propagation of the Ca^{2+} wave was severely attenuated or completely inhibited (Fig. 3A). These results confirm that an increase in $[Ca^{2+}]_i$ is not sufficient to propagate a Ca^{2+} wave to an adjacent cell and are consistent with the passage of IP_3 (or a messenger that stimulates IP_3 production) between adjacent cells.

To demonstrate that intercellular signaling is intact between heparin-loaded cells and heparin-free cells, we applied mechanical stimulation to a heparin-loaded cell at the PHFE border (Fig. 3B). The stimulated cell responded with an increase of $[Ca^{2+}]_i$, and adjacent heparin-free cells responded with a typical Ca^{2+} wave. However, a Ca^{2+} wave did not propagate through the adjacent heparin-loaded cells although an attenuated response in the vicinity of the stimulated cell, inside the heparin-loaded area, was sometimes observed. In cases where heparin-loaded cells showed a response, the increase of $[Ca^{2+}]_i$ occurred after a longer lag time or with a reduced magnitude, or both, as compared to unloaded cells. In addition, the initiation of an $[Ca^{2+}]_i$ increase in heparin-loaded cells sometimes occurred at points other than at the cell borders and in cells not adjacent to cells with high $[Ca^{2+}]_i$ (Fig. 3B, 5 s). This response implies that a messenger other than Ca^{2+} communicates the observed increases in $[Ca^{2+}]_i$. Because the absolute amount of heparin loaded into a cell with PHFE is dependent on size and shape of the cell (*10*), some cells take up less heparin than others. Incomplete blockage of IP_3 receptors might explain the attenuated response seen in the heparin-loaded cells close to the stimulated cell. Mechanical stimulation of cells loaded with TRD or loaded with both TRD and chondroitin sulfate resulted in Ca^{2+} waves that propagated between loaded and unloaded cells (n = 8). These results indicate that heparin prevents the increases in $[Ca^{2+}]_i$ associated with Ca^{2+} waves, but the routes of communication and the mechanism responsible for the initiation of Ca^{2+} waves appear to be unaffected by either heparin or PHFE.

To determine if a depletion of intracellular Ca^{2+} was induced by heparin loading and accounted for the inability of cells to propagate Ca^{2+} waves, we simultaneously observed the responses of heparin-loaded and heparin-free cells to thapsigargin, an inhibitor of intracellular Ca^{2+}-pump activity that prevents sequestering of cytoplasmic Ca^{2+} into IP_3-sensitive intracellular stores (*13–15*). Thapsigargin increased $[Ca^{2+}]_i$ from 36 ± 6 to 336 ± 44 nM (mean ± SEM; n = 52, for four experiments) in heparin-free cells and from 46 ± 9 to 283 ± 33 nM (n = 73) in heparin-loaded cells after 50 s. Under Ca^{2+}-free conditions, thapsigargin increased the $[Ca^{2+}]_i$ from 59 ± 13 nM to a maximal value of 252 ± 28 nM (n = 57, for three experiments) in heparin-free cells and from 43 ± 5 nM to 212 ± 20 nM (n = 67) in heparin-loaded cells in 45 to 60 s. The initial $[Ca^{2+}]_i$ or changes in $[Ca^{2+}]_i$ induced by thapsigargin between heparin-loaded and heparin-free cells were not significantly different; only the increases in $[Ca^{2+}]_i$ of both heparin-loaded and heparin-free cells in Ca^{2+}-free conditions were significantly lower than those in the presence of extracellular Ca^{2+} (*15*). These results are in agreement with reports that emptying of cytoplasmic Ca^{2+} stores results in an influx of Ca^{2+} (*16*) and indicate that the intracellular Ca^{2+} storage capacity of heparin-loaded cells is intact. Mechanical stimulation of thapsigargin-treated cells, even 1 hour after transient exposure to thapsigargin, did not initiate an intercellular Ca^{2+} wave. After treatment with thapsigargin, $[Ca^{2+}]_i$ increased in mechanically stimulated cells in the presence, but not in the absence, of extracellular Ca^{2+}. Similar responses to thapsigargin have been observed in glial cells (5). These results also indicate that an influx of Ca^{2+} contributes to the increase of $[Ca^{2+}]_i$ in the stimulated cell and that IP_3-dependent Ca^{2+} release from intracellular Ca^{2+} pools is necessary for propagation of Ca^{2+} waves.

Cultured epithelial cells underwent spontaneous oscillations in $[Ca^{2+}]_i$ that were not communicated to adjacent cells (Fig. 4). However, mechanically induced Ca^{2+} waves propagated through cells displaying oscillations in $[Ca^{2+}]_i$, even if the communicating cell was undergoing an oscillation in $[Ca^{2+}]_i$ (Fig. 4E). This suggests that the restriction of changes in $[Ca^{2+}]_i$ associated with oscillations is not the result of the closure of gap junctions by increased $[Ca^{2+}]_i$ (*17*). Oscillations in $[Ca^{2+}]_i$ could be of a similar size to increases in $[Ca^{2+}]_i$ induced by Ca^{2+} wave propagation but were still not communicated (Fig. 4). Agonist-induced oscillations of $[Ca^{2+}]_i$ in glial cells can also be larger than increases in $[Ca^{2+}]_i$ resulting from Ca^{2+}-wave propagation (3). These results suggest that a threshold increase in $[Ca^{2+}]_i$ is not required for communication and emphasizes that an in-

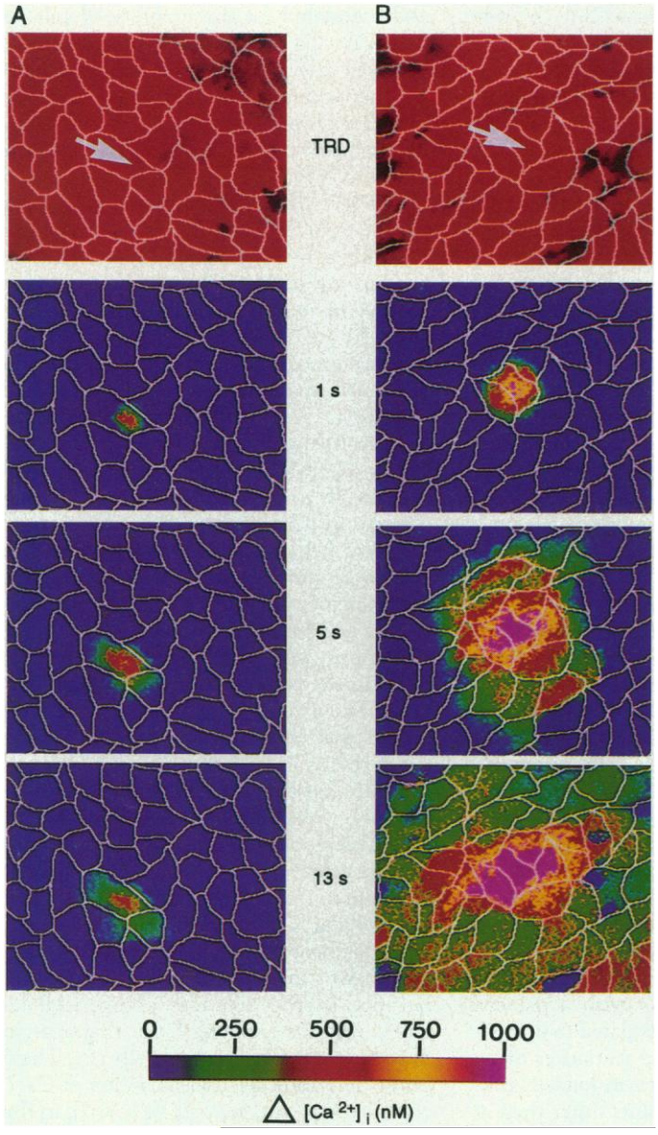

Fig. 2. (left). Blockage of intercellular propagation of Ca^{2+} waves by heparin but not by chondroitin sulfate. Cells cultured 10 to 20 days were loaded by PHFE with either (**A**) heparin or (**B**) chondroitin sulfate (*11*) (Fig. 1); this loading is illustrated by the fluorescence of TRD (top panel) and was similar in both cases. Changes in $[Ca^{2+}]_i$ of the same cells at the indicated times after a mechanical stimulation of a single cell (white arrow) are shown in the lower three panels (*12*). Cell borders are outlined in white. Responses are representative of 18 (A) and 4 (B) experiments. The change (Δ) in $[Ca^{2+}]_i$ from resting concentrations is indicated by the scale bar (*12*). Fields shown are 170 μm (vertical) by 215 μm (horizontal).

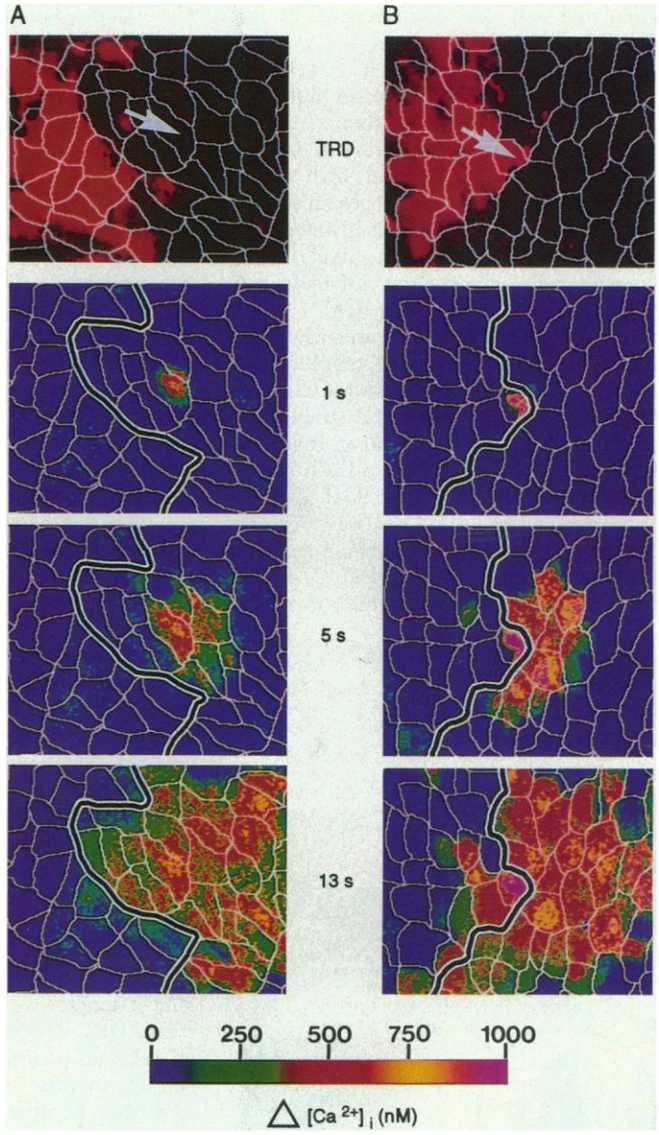

Fig. 3. (right). Propagation of intercellular Ca^{2+} waves out of heparin-loaded cells but not into heparin-loaded cells. PHFE loading of cells with heparin (Fig. 1) (*11*) was detected by the TRD fluorescence (top panel). The boundary of the heparin-loaded cells is indicated by the black and white line. Changes of $[Ca^{2+}]_i$ (lower three panels) at the indicated times after mechanical stimulation (**A**) of a single cell (white arrow) outside the heparin-loaded area and (**B**) of a cell loaded with heparin at the loading boundary (*12*). Responses represent nine (A) and seven (B) experiments. Scale bar and field sizes as in Fig. 2.

crease in $[Ca^{2+}]_i$ alone cannot propagate Ca^{2+} waves; Ca^{2+}-free conditions also induced oscillations in $[Ca^{2+}]_i$ in heparin-loaded cells that did not communicate Ca^{2+} waves (Fig. 4, F through I). Thus, these oscillations appear to occur independently of changes in intracellular IP_3 concentration and may result from Ca^{2+}-induced Ca^{2+} release (*18*), a conclusion consistent with the occurrence of $[Ca^{2+}]_i$ oscillations in hepatocytes and parotid acinar cells depleted of IP_3-dependent Ca^{2+} pools (*14*, *19*).

The propagation of intercellular Ca^{2+} waves is consistent with a model in which mechanical stimulation results in the production of IP_3 in the stimulated cell, which can diffuse across the stimulated cell and through gap junctions to trigger the release of intracellular Ca^{2+} in adjacent cells. The wave of IP_3-induced changes in $[Ca^{2+}]_i$ may also be amplified by Ca^{2+}-induced Ca^{2+} release to form the intracellular Ca^{2+} wave in each cell (*2*, *3*, *5*). Mathematical modeling predicts that diffusion of IP_3 from only the stimulated cell is inadequate to account for the observed patterns of Ca^{2+} wave propagation (*20*). Therefore, a partial regenerative production and local diffusion through gap junctions of IP_3 appear necessary for multicellular communication. However, the possibility remains that a messenger other than IP_3 could be transferred between cells to stimulate an increase in the concentration of IP_3. Intercellular communication between amphibian follicular cells and oocytes also appears to occur by an IP_3-mediated process (*21*).

The physiological significance of $[Ca^{2+}]_i$ oscillations is not understood; $[Ca^{2+}]_i$ oscil-

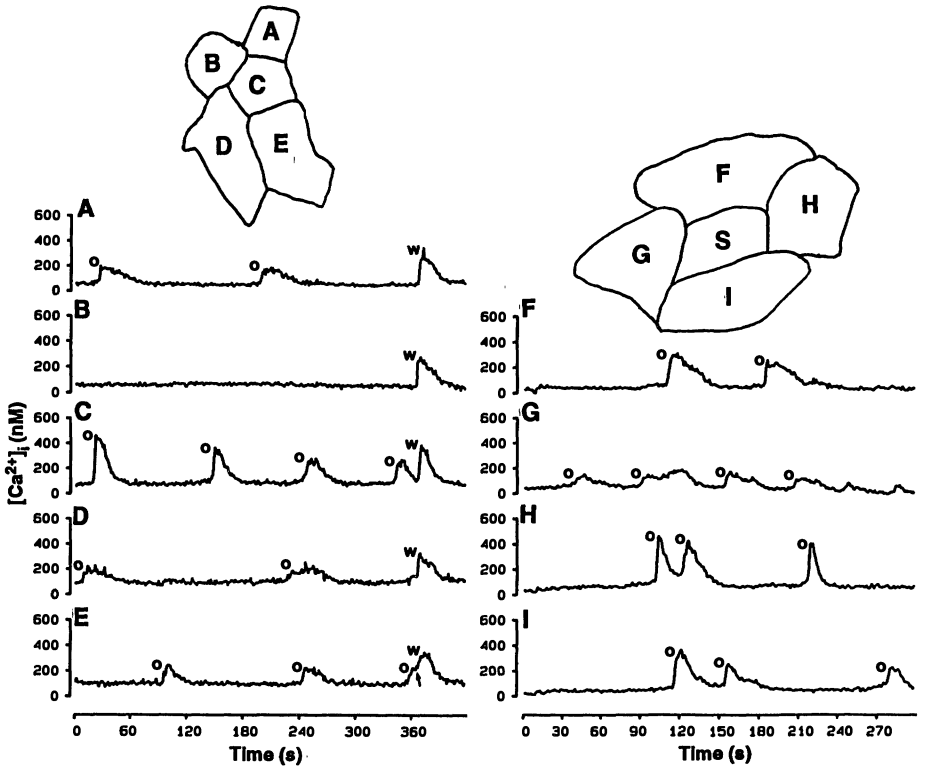

Fig. 4. Asynchronous oscillations in $[Ca^{2+}]_i$ and propagating Ca^{2+} waves in airway epithelial cells. (**A**) through (**E**) Peak $[Ca^{2+}]_i$ reached during spontaneous oscillations (o) in adjacent cells were separated by >20 s and occurred with different periodicities (*12*). By contrast, peak $[Ca^{2+}]_i$ changes induced by a Ca^{2+} wave (w) initiated in a distal cell (not included) propagated through all of these cells in seconds, even during an oscillation of $[Ca^{2+}]_i$ (E, arrow). Cell positions are shown (top panel). (**F**) through (**I**) Oscillations of $[Ca^{2+}]_i$ in heparin-loaded cells after exposure to Ca^{2+}-free solution. Before inducement of oscillations, we mechanically stimulated cell S. Despite increase of $[Ca^{2+}]_i$ in cell S after stimulation, cells F through I showed no increase in $[Ca^{2+}]_i$ indicating that blockage of the Ca^{2+} wave by heparin was complete.

lations may be a form of frequency-modulated signaling that enhances signal recognition and avoids desensitization and Ca^{2+} toxicity (*4*) or a mechanism that allows for refilling Ca^{2+} stores (*22*). Alternatively, Ca^{2+} oscillations may drive specific cellular functions, such as directional ion transport across cells (*23*). We suggest that the asynchronous nature of $[Ca^{2+}]_i$ oscillations indicates that adjacent cells are acting independently of one another. By contrast, in airway cells, propagating Ca^{2+} waves increase ciliary beat frequency in a localized group of cells, and this could enhance mucociliary clearance (*24*). Consequently, we suggest that IP_3-dependent intercellular Ca^{2+} waves provide a mechanism of intercellular signaling that coordinates or initiates multicellular activity.

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11. Cells were washed three times in electroporation medium (EPM) [300 mM sorbitol, 2.0 mM Hepes, 4.2 mM KH_2PO_4, 10.8 mM K_2HPO_4 (pH 7.2), and 1.0 mM $MgCl_2$] with no added Ca^{2+}. Cells were electroporated with a pulsed, high-frequency electric field in EPM containing 75 μM TRD (10 kD; Molecular Probes, Eugene, OR) and heparin (2 mg/ml) (H-9133, Sigma) or chondroitin sulfate (3 mg/ml) (C-8529, Sigma). The electric field consisted of a 65-kHz triangular wave of 1200 to 1300 V/cm in three trains of ten 1-ms pulses with two 10-s break periods. After PHFE, cells were washed several times with Hanks' buffered saline solution (HBSS) without phenol red (310-4025, Gibco-BRL), additionally buffered with 25 mM Hepes (pH 7.4).
12. Image analysis of fura-2 fluorescence and construction of $[Ca^{2+}]_i$ maps were performed as described (*2*, *3*). After PHFE, cells were incubated in 5 μM fura-2-AM (Calbiochem) in HBSS (*11*) at 21° to 24°C for 50 to 60 min, washed in HBSS, and used after 30 min. $[Ca^{2+}]_i$ was calculated with ratiometric methods (*25*) and, when increased time resolution was necessary (1/30 s), from single-wavelength recordings with reference to an initial $[Ca^{2+}]_i$ map calculated ratiometrically (*3*). Both methods yielded similar values for $[Ca^{2+}]_i$. Plots of $[Ca^{2+}]_i$ versus time are of an area at the approximate center of the cell covering 2.6 μm by 2.1 μm. Individual points plotted are averages of data from four consecutive video frames (taken at 30 frames per second) at intervals of 1 s. Images of $[Ca^{2+}]_i$ represent the change in $[Ca^{2+}]_i$ from resting $[Ca^{2+}]_i$ (1 s before mechanical stimulation).
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15. Cells were washed with 2 ml of 1 μM thapsigargin in Ca^{2+}-containing (HBSS) (*11*) or Ca^{2+}-free solution consisting of 25 mM Hepes (pH 7.2), 5.0 mM KCl, 0.3 mM KH_2PO_4, 2.0 mM $MgCl_2$, 140 mM NaCl, 0.3 mM Na_2HPO_4, 4.0 mM $NaHCO_3$, and 1.0 mM EGTA. Cells retained TRD and fura-2 fluorescence and displayed ciliary activity for at least 1 hour after addition of thapsigargin in HBSS. A two-sample *z* statistic with a rejection region defined by $\alpha = 0.05$ was used for statistical analyses.
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