Intracellular calcium oscillations induced by ATP in airway epithelial cells

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Evans, John H., and Michael J. Sanderson. Intracellular calcium oscillations induced by ATP in airway epithelial cells. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L30–L41, 1999.—In airway epithelial cells, extracellular ATP (ATP₀) stimulates an initial transient increase in intracellular Ca²⁺ concentration that is followed by periodic increases in intracellular Ca²⁺ concentration (Ca²⁺ oscillations). This leads to the production of inositol 1,4,5-trisphosphate; calcium waves; uridine 5'-triphosphate; adenosine 5'-triphosphate; cell signaling.

Oscillatory changes in intracellular Ca²⁺ concentration ([Ca²⁺]) or Ca²⁺ oscillations, occur in a variety of nonexcitable cell types (47). Commonly, Ca²⁺ oscillations arise after the activation of phospholipase C (PLC) by agonists binding to cell surface receptors (1). This leads to the production of inositol 1,4,5-trisphosphate ([IP₃]) followed by Ca²⁺ mobilization from internal stores via the IP₃ receptor (IP₃R), itself a Ca²⁺ channel, and to an increase in [Ca²⁺]. Ca²⁺ feedback inhibition of the IP₃R results in cessation of Ca²⁺ release (3), and this together with the sequestration and extrusion of Ca²⁺ from the cytoplasm by pumps into the endoplasmic reticulum or across the plasma membranes, respectively, leads to a decline in [Ca²⁺]. Once the [Ca²⁺] falls to a permissive level, repetitive cycles of [Ca²⁺] increase and decline are maintained by an elevated intracellular IP₃ concentration ([IP₃]) (13, 15). Thus Ca²⁺ oscillations are dependent on the action of both [Ca²⁺], and [IP₃], on the IP₃R. Experimental data show that the frequency of Ca²⁺ oscillations are dependent on [IP₃], and, indirectly, agonist concentration (15). Mathematical modeling of Ca²⁺ oscillations as a function of [IP₃] and [Ca²⁺] agrees with the experimental findings (43).

Extracellular ATP (ATP₀) serves as an agonist in many organs and tissues (12) and induces an initial transient increase in [Ca²⁺], followed by Ca²⁺ oscillations in a number of cell types including astrocytes (49), smooth muscle cells (24), granulosa luteal cells (44), Madin-Darby canine kidney (MDCK) cells (35), chondrocytes (8), bile duct epithelial cells (29), and megakaryocytes (46). In airway cells, ATP₀ mobilizes [Ca²⁺], via PLC by activating P2U receptors (12). P2U receptors are expressed at the apical pole of rat airway epithelial cells and respond to extracellular UTP (UTP₀) as well as to ATP₀ (19, 26). ATP₀ and UTP₀ may serve as important physiological factors in the airway lumen because airway cells are reported to release ATP and UTP in response to stretch (14) and via the cystic fibrosis transmembrane conductance regulator (9). Released ATP₀ or UTP₀ may act in an autocrine or paracrine fashion to mediate Cl⁻ secretion (42) and, possibly, ciliary beat frequency (16, 50).

The spatial organization of [Ca²⁺] responses to an agonist are also important in coordinating Ca²⁺-mediated activity at the level of tissues or organs. For example, synchronous Ca²⁺ oscillations have been reported in tissues such as pancreatic acini (45), islets of Langerhans (2), lung capillary endothelium (51), and liver lobules (30, 33) and in cultures of hepatocytes (48), MDCK cells (35), and chondrocytes (8). The synchrophone of Ca²⁺ oscillations in the above systems invariably relies on gap junction communication between contacting cells, and the synchrophone is lost when communication is disrupted. In perfused pancreatic acini (45) and other confluent cell cultures, such as arterial smooth muscle cells (24) and MDCK cells (35), however, no synchrophone of Ca²⁺ oscillations between contacting cells is observed. Thus between cell types and experimental conditions, the spatial coordination of Ca²⁺ oscillations differs. These differences may then be exploited to investigate different mechanisms of intercellular communication. Because both Ca²⁺ and IP₃ can act as intercellular messengers (36), it remains unclear whether Ca²⁺ or IP₃ is the messenger communicated though the gap junctions to initiate intercellular Ca²⁺ waves or whether the intercellular messenger em-

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employed is cell-type or condition specific. In addition to gap junction communication, cell messengers can be communicated extracellularly (20, 41). In primary cultures of airway epithelial cells, the intercellular diffusion of IP₃ is consistent with the observed propagation of intercellular Ca²⁺ waves under a number of experimental conditions (5).

Although Ca²⁺-sensitive cell functions are often mediated by oscillatory rather than prolonged sustained increases in [Ca²⁺] (32), only a few brief reports have shown Ca²⁺ oscillations in ciliated airway epithelial cells (21, 31, 37), goblet cells (31), or airway gland cells (25). Using digital microscopy techniques and the Ca²⁺ indicator fura 2, we investigated the temporal aspects of the [Ca²⁺] response to ATPₒ and UTPₒ in airway epithelial cells and describe here the mechanism by which Ca²⁺ oscillations are initiated and maintained. We found that ATP-induced Ca²⁺ oscillations in airway epithelial cells were dependent on the ATPₒ concentration ([ATPₒ]), were mediated by G protein-coupled receptors involving an IP₃ signaling pathway, and were not communicated to adjacent cells. Thus airway epithelial cells exhibit two fundamental modes of Ca²⁺ signaling, intracellular Ca²⁺ oscillations and intercellular Ca²⁺ waves, which may occur simultaneously within a cell.

MATERIALS AND METHODS

The techniques for culturing airway epithelial cells, mechanically stimulating individual cells, and the measurement of [Ca²⁺] by fluorescent video microscopy have been described in detail elsewhere (7, 23, 38-40) and will be only briefly reviewed here.

Cell culture. Primary cultures of airway epithelial cells were prepared from the trachea of New Zealand White rabbits as previously described (11) except that the collagen on the coverslips was fixed with formaldehyde instead of glutaraldehyde. After dissection of the epithelial mucosa from the trachea, the tissue was cut into ~0.5 mm squares, placed onto collagen-coated coverslips, and cultured in DMEM supplemented with 10% fetal bovine serum, 10 mM HEPES, and antibiotics/antimycotics at 37°C in 10% CO₂ for 5–9 days.

Measurement of [Ca²⁺]. Cells were loaded with fura 2 by incubation at 37°C for 1 h in 5 µM fura 2-AM (Calbiochem) in Ca²⁺-free experiments, Ca²⁺/Mg²⁺-free Dulbecco’s phosphate-buffered saline (DPBS; Gibco BRL) and additionally buffered with 25 mM HEPES (HHBSS, pH 7.4). The cells were washed and allowed to incubate at room temperature for 30 min to allow for complete deesterification of the fura 2-AM. In Ca²⁺-free experiments, Ca²⁺/Mg²⁺-free Dulbecco’s phosphate-buffered saline (DPBS; Gibco BRL) was used in place of HHBSS. Cells were visualized with a Nikon inverted microscope equipped with fluorescence optics and a ×40 objective lens. Fluorescence was detected with a silicon-intensified target camera, recorded with an optical memory disk recorder, and digitized by computer (7, 38). Images of [Ca²⁺] were calculated by single-wavelength recordings referenced to ratiometric measurements (23). Initial [Ca²⁺], reference images were based on 10 frames recorded at 340 and 380 nm. Changes in [Ca²⁺], were recorded by monitoring changes in fluorescence with an illumination wavelength of 380 nm. Additional reference images were taken at 340 nm every 30 s. [Ca²⁺] was calculated from the change in fluorescence intensity at 380 nm (7, 23). All images were subjected to background subtraction and correction for shading and bleaching.

RESULTS

Response to ATPₒ. The Ca²⁺ response of airway epithelial cells to ATPₒ was quantified with digital video microscopy in confluent primary cell cultures (5–9 days old) loaded with the Ca²⁺ indicator fura 2. The application of ATPₒ (0.1–100 µM) induced an initial Ca²⁺ transient in >90% of cells in the field of view. However, the magnitude of this [Ca²⁺] increase varied greatly from cell to cell, but in general, it increased with increasing [ATPₒ]. After the initial [Ca²⁺] increase induced by ATPₒ, 7–36% of all the cells, depending on the [ATPₒ], displayed periodic Ca²⁺ oscillations (Fig. 1). These Ca²⁺ oscillations ceased immediately on ATPₒ washout (data not shown). The greatest number of cells displayed Ca²⁺ oscillations in response to 5 µM ATP (n = 386–503 cells, 3–4 experiments; Fig. 2A). These Ca²⁺ oscillations were initiated 15 s to several minutes after the initial [Ca²⁺] transient and consisted of a sharp increase in [Ca²⁺] followed by a slower [Ca²⁺] decline; however, the [Ca²⁺] increases of the Ca²⁺ oscillations were smaller than that of the initial Ca²⁺ transient. In many experiments, the initial two to three Ca²⁺ oscillations were characterized by a higher frequency and were initiated from a higher baseline [Ca²⁺] than subsequent oscillations.

Frequently, each Ca²⁺ oscillation occurred as an intracellular Ca²⁺ wave that spread across the individual cell. The region from which the Ca²⁺ wave initiated and the direction of the wave propagation was constant for each intracellular Ca²⁺ oscillation and was unique to each individual cell. The relationship of the Ca²⁺ wave initiation site to a specific cell organelle or structure was not determined but may result from the heterogeneity in the distribution and sensitivity of the
Ca\(^{2+}\) pools or IP3Rs. This relationship will be the subject of a future study. Simultaneous phase-contrast imaging also demonstrated that the ciliary beat frequency of the cells increased and decreased in concert with the [Ca\(^{2+}\)]\(_i\) changes of each Ca\(^{2+}\) oscillation, and studies correlating the [Ca\(^{2+}\)]\(_i\) and ciliary beat frequency changes also need to be completed.

Dose-response experiments revealed that individual cells within a population exhibited differing sensitivities to ATP\(_o\) and that this determined the characteristics of the Ca\(^{2+}\)-oscillatory behavior of each cell. Cells could be categorized as having a high, intermediate, or low sensitivity to ATP\(_o\). Within each category, four basic responses to ATP\(_o\) were well identified (Fig. 1). These consisted of 1) a single Ca\(^{2+}\) transient after exposure to ATP\(_o\); 2) the initiation of a few irregular Ca\(^{2+}\) oscillations; 3) the initiation of well-defined, multiple Ca\(^{2+}\) oscillations; and 4) a prolonged elevation of the baseline [Ca\(^{2+}\)]\(_i\) after the initial spike. For each cell, this range of responses required a 5- to 10-fold increase in ATP\(_o\). For example, a cell with high sensitivity to ATP\(_o\) (Fig. 1A) responded to 0.5 or 1 µM ATP with regular, periodic Ca\(^{2+}\) oscillations. However, the same cell responded to 5 µM ATP with an initial [Ca\(^{2+}\)]\(_i\) transient followed by a prolonged elevated [Ca\(^{2+}\)]\(_i\) without any subsequent Ca\(^{2+}\) oscillations. By contrast, a cell with an intermediate sensitivity to ATP\(_o\) (Fig. 1B) responded to 5 µM ATP with well-defined Ca\(^{2+}\) oscillations and required 50 µM ATP to induce an elevated baseline of [Ca\(^{2+}\)]\(_i\) without Ca\(^{2+}\) oscillations. On the other hand, a cell with low sensitivity to ATP\(_o\) required 50 µM ATP to induce regular Ca\(^{2+}\) oscillations (Fig. 1C).

These differing Ca\(^{2+}\)-oscillatory responses to ATP\(_o\) are summarized in Fig. 2. The greatest number of cells (46% of the culture) was classified as having an intermediate sensitivity to ATP\(_o\) and these cells displayed the greatest number of Ca\(^{2+}\) oscillations in response to 10 µM ATP. Cells classified as having either a high (32% of the culture) or low sensitivity (22% of the culture) to ATP\(_o\) displayed the greatest number of Ca\(^{2+}\) oscillations in response to 1 or 100 µM ATP, respectively. Therefore, in a single culture, individual cells displayed similar [Ca\(^{2+}\)]\(_i\) responses to ATP\(_o\) stimulation but over a 100-fold concentration range.

Response to UTP\(_o\). In an earlier study by Hansen et al. (16), the [Ca\(^{2+}\)]\(_i\) response of tracheal epithelial cells to ATP\(_o\) was blocked by the antagonist suramin, suggesting P2 purinergic-receptor involvement. Because P2U receptors exhibit a greater or equal selectivity for UTP\(_o\) over ATP\(_o\) (12), we determined the [Ca\(^{2+}\)]\(_i\) response of cultured epithelial cells to UTP\(_o\). The [Ca\(^{2+}\)]\(_i\) response of individual cells to 0.1 µM UTP\(_o\) or ATP\(_o\) is shown in Fig. 3. In one cell (Fig. 3A), UTP\(_o\) elicited an initial [Ca\(^{2+}\)]\(_i\) transient, whereas ATP\(_o\) had no effect on the [Ca\(^{2+}\)]. In another cell (Fig. 3B), the initial [Ca\(^{2+}\)]\(_i\) transient in response to UTP\(_o\) was followed by a single Ca\(^{2+}\) oscillation, whereas ATP\(_o\) elicited only an initial
increase in response to 0.1 µM ATP (n = 229 cells, 4 experiments; Fig. 4A). Less than 2% of cells exhibited an [Ca\(^{2+}\)] increase in response to ATP\(_o\), but not UTP\(_o\). Also, 0.1 µM UTP initiated Ca\(^{2+}\) oscillations in 59% of cells compared with 40% with 0.1 µM ATP (Fig. 4A), and the cells stimulated with UTP\(_o\) showed a greater number of oscillations than those stimulated with ATP\(_o\) (Fig. 4B). These responses to ATP\(_o\) or UTP\(_o\) were independent of the order of agonist challenge.

Fig. 2. Summary of Ca\(^{2+}\) response of airway epithelial cells to ATP\(_o\). A: airway epithelial cells displayed greatest Ca\(^{2+}\) oscillatory behavior at 5 µM ATP. [ATP]o, ATP concentration. B: in a single culture, average number of Ca\(^{2+}\) oscillations/cell (defined as average number of [Ca\(^{2+}\)] increases/cell during a 5-min recording period after initial [Ca\(^{2+}\)] increase) increased with increasing [ATP]o values. However, [ATP]o value generating the greatest number of Ca\(^{2+}\) oscillations/cell was dependent on ATP\(_o\) sensitivity of cell. C: no. of cells in each ATP sensitivity category in a single cell culture (same culture as in B). Intermed, intermediate. Data in A are from 388–503 cells from 3–5 experiments analyzed at each [ATP]o value. Data in B and C are from a single experiment with 63 cells analyzed.

Fig. 3. Differential response of individual cells to extracellular UTP (UTP\(_o\)) and ATP\(_o\). Cultured cells were sequentially exposed to 0.1 µM ATP and 0.1 µM UTP or vice versa. Traces of [Ca\(^{2+}\)] change over time are representative of individual cells in which UTP\(_o\) but not ATP\(_o\) elicited an initial [Ca\(^{2+}\)] increase (A) or initiated a Ca\(^{2+}\) oscillation subsequent to the initial [Ca\(^{2+}\)] increase (B). C: trace is representative of cells in which UTP\(_o\) initiated a higher frequency of Ca\(^{2+}\) oscillations compared with that initiated by ATP\(_o\). Traces are representative of 229 cells analyzed from 4 experiments.
Ca$^{2+}$ oscillations and the release of internal Ca$^{2+}$ stores. IP$_3$-mediated Ca$^{2+}$ oscillations typically are dependent on Ca$^{2+}$ release from internal stores and are independent of the extracellular Ca$^{2+}$ concentration ([Ca$^{2+}]_{o}$). To investigate the dependence of ATP-induced Ca$^{2+}$ oscillations on [Ca$^{2+}]_{i}$, cells were stimulated with 5 µM ATP in Ca$^{2+}$/Mg$^{2+}$-free DPBS. Because extracellular unbound EGTA has been shown to interfere with Ca$^{2+}$ release from intracellular stores in airway epithelial cells (18) and because the Ca$^{2+}$ response to histamine in airway cells was essentially the same in nominally Ca$^{2+}$-free medium, medium containing 1 mM 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid, and medium with an [Ca$^{2+}]_{o}$ of ~7 nM (18), we decided to use a nominally Ca$^{2+}$-free medium for these experiments. Ca$^{2+}$ oscillations induced by ATP$_{o}$ occurred in the absence of extracellular Ca$^{2+}$ (n = 33 cells, 3 experiments; Fig. 6A). Treatment with Ca$^{2+}$/Mg$^{2+}$-free DPBS alone failed to induce Ca$^{2+}$ oscillations (Fig. 6B).

To investigate further the dependence of Ca$^{2+}$ oscillations on internal Ca$^{2+}$ stores, we used the Ca$^{2+}$-ATPase inhibitor thapsigargin. Prolonged incubation (20 min) of cells with 1 µM thapsigargin elevated the baseline Ca$^{2+}$ by 10.22 ± 0.32. U-73122 was added to cells exhibiting ongoing ATP-induced Ca$^{2+}$ oscillations. ATP-induced Ca$^{2+}$ oscillations quickly ceased on addition of U-73122 (Fig. 5A). The addition of U-73343 had no effect on ongoing Ca$^{2+}$ oscillations (n = 83 cells, 3 experiments; Fig. 5B).

Fig. 4. Summary of differential responses to ATP$_{o}$ and UTP$_{o}$. A: of the cells which exhibited at least 1 [Ca$^{2+}]_{i}$ increase in response to 0.1 µM ATP, 91% also responded to 0.1 µM UTP. Only 4 of 229 cells from 4 experiments that responded to ATP$_{o}$ failed to respond to UTP$_{o}$. Of the responding cells, 99% of cells exhibited oscillations in ATP$_{o}$ whereas only 40% exhibited oscillations in ATP$_{o}$. B: average no. of oscillations/ cell induced by UTP$_{o}$ was higher than that induced by ATP$_{o}$ in the 5-min recording period.

Fig. 5. Effect of phospholipase C inhibitor U-73122 and an inactive analog, U-73343, on [Ca$^{2+}]_{i}$ changes induced by ATP$_{o}$. A: U-73122 was added to cells 2 min after exposure to 5 µM ATP. Trace shows that ATP$_{o}$ induced a characteristic Ca$^{2+}$ oscillation pattern and that Ca$^{2+}$ oscillations ceased after addition of U-73122. B: similar experiment shows that U-73343 had no effect on Ca$^{2+}$ oscillations (n = 83 cells from 3 experiments).
[Ca^{2+}]i in the cells and abolished the Ca^{2+} response of the cells to 5 µM ATP (data not shown). Again, Ca^{2+} oscillations may be dependent on an initial Ca^{2+} increase but independent of internal Ca^{2+} pools. To investigate this possibility, cells were exposed for a short time (1 min) to thapsigargin before stimulation with ATPo. This short-term exposure to thapsigargin stimulated a small rise in [Ca^{2+}]i but allowed a large ATP-induced [Ca^{2+}]i increase (n = 91 cells, 4 experiments; Fig. 7A). Baseline [Ca^{2+}]i levels remained elevated in these cells, and the cells failed to exhibit Ca^{2+} oscillations. An identical treatment of cells with DMSO, the vehicle for thapsigargin, resulted in a normal Ca^{2+} response to ATPo treatment (Fig. 7B). To determine whether internal release of Ca^{2+} is the sole mechanism driving ATP-induced Ca^{2+} oscillations, we treated cells displaying ATP-induced Ca^{2+} oscillations with thapsigargin. After the addition of thapsigargin to Ca^{2+}-oscillating cells, the amplitude of the Ca^{2+} oscillations decreased and the baseline [Ca^{2+}]i increased with each successive oscillation until oscillations ceased (n = 154 cells, 5 experiments; Fig. 7, C and D).

Spatial characteristics of Ca^{2+} oscillations. To ascertain the ability of cells exhibiting Ca^{2+} oscillations to influence the Ca^{2+} activity of neighboring cells, we analyzed the temporal and spatial [Ca^{2+}]i responses of cells to ATPo in confluent cultures. Pseudocolor images of the [Ca^{2+}]i change in airway epithelial cells exposed to 5 µM ATP (Fig. 8) showed that after an initial [Ca^{2+}]i increase stimulated by the addition of ATPo (Fig. 8A), many cells displayed repetitive increases in [Ca^{2+}]i of differing frequency and amplitude (Fig. 8, B–I). The asynchronous nature of the Ca^{2+} oscillations is exemplified by five adjacent cells (Fig. 8, u) that exhibited Ca^{2+} oscillations independently from one another.

Graphic representations of the [Ca^{2+}]i changes in four adjacent cells (Fig. 9) show that all cells responded to the addition of 5 µM ATP with a nearly simultaneous [Ca^{2+}]i increase. However, the patterns of Ca^{2+} oscillations differed greatly between cells. For example, the...
The first \( \text{Ca}^{2+} \) oscillation in cell B (central cell) occurred at \( \sim 40 \text{ s} \) (Fig. 9, line 1) and failed to propagate to cell C. This \( \text{Ca}^{2+} \) oscillation preceded the first \( \text{Ca}^{2+} \) oscillation of cell A by \( \sim 50 \text{ s} \) and followed the first \( \text{Ca}^{2+} \) oscillation of cell D by \( \sim 8 \text{ s} \). Similarly, the second \( \text{Ca}^{2+} \) oscillation in cell B (Fig. 9, line 2) preceded the first \( \text{Ca}^{2+} \) oscillation in cell A by \( \sim 6 \text{ s} \) and followed the second \( \text{Ca}^{2+} \) oscillation in cell D by \( \sim 20 \text{ s} \). By 150 s, cells A–D have experienced 1, 3, 0, and 4 \( \text{Ca}^{2+} \) oscillations, respectively.

**Intercellular \( \text{Ca}^{2+} \) signaling.** In a previous study (6), it has been demonstrated that in response to mechanical stimulation, airway epithelial cells propagated increases in \([\text{Ca}^{2+}]_i\) similar to those associated with \( \text{Ca}^{2+} \) oscillations to adjacent cells as intercellular \( \text{Ca}^{2+} \) waves. The mechanism responsible for the intercellular waves was proposed to be the diffusive spread of IP\(_3\) rather than of \( \text{Ca}^{2+} \) between adjacent cells via gap junctions. Consequently, the asynchronous nature and failure of ATP-induced \( \text{Ca}^{2+} \) oscillations to influence the \( \text{Ca}^{2+} \) activity of adjacent cells may result either from an inherent but independent regulation of cell function or, alternatively, from a lack of gap junction communication between cells. To explore this hypothesis and to estimate the extent of cell-cell communication, the ability of cells displaying ATP-induced \( \text{Ca}^{2+} \) oscillations to propagate intercellular \( \text{Ca}^{2+} \) waves was tested.

Figure 10 shows pseudocolor images of the change in \([\text{Ca}^{2+}]_i\) in confluent cells that have been exposed to 5 \( \mu \text{M ATP} \) and have been subsequently mechanically stimulated. In response to ATP\(_o\), many cells exhibit \( \text{Ca}^{2+} \) oscillations (Fig. 10, A–C, \(*\)). Because of the variability in sensitivity of individual cells to ATP\(_o\), the single dose of ATP\(_o\) evoked differing \( \text{Ca}^{2+} \) responses in adjacent cells. After mechanical stimulation of a single cell (Fig. 10D, arrow), an intercellular \( \text{Ca}^{2+} \) wave spread through \( \text{Ca}^{2+} \)-oscillating and nonoscillating cells (Fig. 10, E and F). After passage of the intercellular \( \text{Ca}^{2+} \) wave (Fig. 10, G–I), many cells (\(*\)) resumed \( \text{Ca}^{2+} \) oscillations.

The traces in Fig. 11 show typical \([\text{Ca}^{2+}]_i\) responses of several adjacent cells after application of ATP\(_o\); the
variability of the Ca^{2+} response is clearly demonstrated in the traces. Cell B shows a response typical for a cell with a high sensitivity to ATP_{o}, whereas cells A, C, and D show responses typical for cells with an intermediate sensitivity. Although the Ca^{2+} response of cells A, C, and D are fundamentally similar, these adjacent cells clearly display Ca^{2+} oscillations of different frequencies and magnitudes, and this emphasizes the asynchronous nature of adjacent cell Ca^{2+} oscillations. At 120 s, mechanical stimulation (Fig. 11, line S) of a single cell (cell A) initiated the propagation of an intercellular Ca^{2+} wave that spread throughout the cell culture, passing through both Ca^{2+}-oscillating and nonoscillating cells. The mechanical stimulation of cell A resulted in an almost immediate and sustained [Ca^{2+}]_{i} increase in that cell. With a very short time delay (~1 s), the [Ca^{2+}]_{i} in cell B rapidly increased and subsequently decreased to a higher baseline [Ca^{2+}]. In cell C, the [Ca^{2+}]_{i} increased after a ~3-s delay to a higher amplitude than the preceding oscillatory [Ca^{2+}]_{i} increases. Interestingly, the next two Ca^{2+} oscillations initiated after a shorter period and at a higher baseline [Ca^{2+}], than did the Ca^{2+} oscillations preceding the intracellular wave. In cell D, after a ~5-s delay, the [Ca^{2+}]_{i} increase in the intercellular Ca^{2+} wave was higher than the initial [Ca^{2+}]_{i} increase; however, it failed to reinitiate Ca^{2+} oscillations.

**DISCUSSION**

In this study, we demonstrated by digital imaging fluorescence microscopy that airway epithelial cells exhibit Ca^{2+} oscillations in response to ATP_{o}. Although Ca^{2+} oscillations have been briefly reported in airway epithelial cells from four species (21, 25, 31, 37), no in-depth characterization has been made of the phenomenon. Here, we analyzed the mechanism underlying the generation of the ATP-induced Ca^{2+} oscillations, the intercellular heterogeneity of ATP_{o} responsiveness, and the intercellular signaling associated with ATP-induced Ca^{2+} oscillations.

Signaling pathway involved in the generation of Ca^{2+} oscillations. Airway epithelial cells exhibit a suramin-sensitive [Ca^{2+}]_{i} response to ATP_{o} (16), which suggests that P2 purinergic receptors are involved in the [Ca^{2+}]_{i} response observed in these studies. Airway epithelial cells have been reported to express several ATP-sensitive purinoceptors, including P2U, P2T, P2Y (19), and P2X (22). The P2X receptor/channel tentatively identified in rabbit ciliated epithelium (22) required low (150 µM) [Ca^{2+}]_{o} for activity, was not active at high (1.5 mM) [Ca^{2+}]_{o}, and was not activated by UTP_{o} (22). Because our experiments were conducted in ~1.3 mM [Ca^{2+}]_{o}, we believe that P2X-receptor activity did not contribute to the [Ca^{2+}]_{i} increases observed. UTP_{o} is ineffective in stimulating P2T and P2X receptors and is much less potent than ATP_{o} in stimulating P2Y receptors but is of equal or greater potency to ATP_{o} in stimulating P2U receptors (12). Consequently, the fact that UTP_{o} elicited a more robust [Ca^{2+}]_{i} response from cells (greater number of cells displaying Ca^{2+} oscillations and a greater number of Ca^{2+} oscillations per cell) than ATP_{o} at equimolar concentrations suggests the involvement of P2U receptors. Cl− secretion in rat airway epithelial cells, attributed to apically located P2U receptors, displayed a similar enhancement by UTP_{o} (19). In addition, ATP released from the cystic fibrosis transmembrane conductance regulator is thought to be involved in the autocrine regulation of Ca^{2+}-dependent Cl− channels via apically located P2U receptors in human airway epithelial cells (10, 42).

The steroidal U-73122, a PLC antagonist, prevented an initial [Ca^{2+}]_{i} increase in response to ATP_{o}, consistent with an earlier report suggesting that the Ca^{2+} response to ATP_{o} was mediated by a PLC/IP_{3} pathway (17). U-73122, when added to cells experiencing ATP-induced Ca^{2+} oscillations, resulted in the immediate cessation of Ca^{2+} oscillations. This indicates...
that both the initial Ca\textsuperscript{2+} increase and the subsequent Ca\textsuperscript{2+} oscillations utilize common signaling elements and that Ca\textsuperscript{2+} oscillations require continuous PLC activity for their maintenance. In addition, when ATP\textsubscript{o} is removed from cells displaying ATP-induced Ca\textsuperscript{2+} oscillations, the oscillations immediately cease, suggesting that continuous receptor signaling is also required for the maintenance of Ca\textsuperscript{2+} oscillations. These experiments, however, cannot distinguish between a cyclic increase in [IP\textsubscript{3}]\textsubscript{i} produced by the positive feedback of Ca\textsuperscript{2+} on Ca\textsuperscript{2+}-sensitive PLC as proposed by the cross-coupling model of Ca\textsuperscript{2+} oscillations (27) and the steady-state increase in [IP\textsubscript{3}]\textsubscript{i} that drives Ca\textsuperscript{2+} oscillations as described in the Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release model (10).

However, the propagation of intercellular waves in airway epithelial cells does not appear to involve the regenerative increase in IP\textsubscript{3}, which would be expected to occur if Ca\textsuperscript{2+}-sensitive PLC isoforms were present in the cell. Hence we believe that the activation of PLC by ATP\textsubscript{o} binding to its receptor results in the steady-state increase in [IP\textsubscript{3}].

IP\textsubscript{3}-mediated Ca\textsuperscript{2+} oscillations usually rely on the repetitive release of Ca\textsuperscript{2+} from internal Ca\textsuperscript{2+} pools, and when airway epithelial cells were exposed to ATP\textsubscript{o} in nominally Ca\textsuperscript{2+}-free medium, the cells displayed characteristic Ca\textsuperscript{2+} oscillations for a number of minutes. These results indicate a dependence on internal Ca\textsuperscript{2+} stores for the oscillations. Refilling of the internal Ca\textsuperscript{2+} stores is mediated by the action of Ca\textsuperscript{2+}-ATPases, which can be inhibited by thapsigargin. Airway epithelial cells treated for 60 s with thapsigargin showed a slight increase in the [Ca\textsuperscript{2+}]\textsubscript{i} during thapsigargin treatment; however, when ATP\textsubscript{o} was applied, the cells responded with a large initial [Ca\textsuperscript{2+}] increase that remained elevated and failed to exhibit Ca\textsuperscript{2+} oscillations. Thus no ATP-induced Ca\textsuperscript{2+} oscillations were observed after discharge of the internal pools when the pool was not allowed to refill. Furthermore, by applying thapsigargin 60 s after the addition of ATP and the onset of Ca\textsuperscript{2+} oscillations, the amplitude of the Ca\textsuperscript{2+} oscillations was progressively diminished and the baseline [Ca\textsuperscript{2+}] was increased until the Ca\textsuperscript{2+} oscillations ceased at an
Characteristics of Ca\textsuperscript{2+} oscillations. Four basic responses to ATP\textsubscript{o} were identified in this study. These were 1) a single Ca\textsuperscript{2+} increase after ATP\textsubscript{o} stimulation; 2) the initiation of a few irregular Ca\textsuperscript{2+} oscillations; 3) the initiation of regular, periodic Ca\textsuperscript{2+} oscillations; and 4) a sustained elevation in the baseline [Ca\textsuperscript{2+}]i after an initial Ca\textsuperscript{2+} increase. The heterogeneity of the Ca\textsuperscript{2+} response in airway epithelial cells was very similar to the heterogeneous Ca\textsuperscript{2+} responses to ATP\textsubscript{o} reported in glial cells (49), bile duct cells (29), megakaryocytes (46), and chondrocytes (8). In airway epithelial cells, however, these four Ca\textsuperscript{2+} responses were reproduced in individual cells exposed to different [ATP]o values. Therefore, the heterogeneity of the Ca\textsuperscript{2+} response was due in part to a 100-fold difference in the sensitivity of individual cells to ATP\textsubscript{o} and allowed us to characterize individual cells as having low, intermediate, and high sensitivities to ATP\textsubscript{o}. The basis of this differential sensitivity was not determined but may arise from variations in the expression, density, or sensitivity of the ATP receptor (P2U), IP\textsubscript{3}Rs, or other components of the signaling pathway. Although these differences may result in different resting [Ca\textsuperscript{2+}]i values, a correlation between ATP sensitivity and resting [Ca\textsuperscript{2+}]i was not found. This suggests that the ATP sensitivity may only be manifested after stimulation.

Ca\textsuperscript{2+}-signaling differences may also arise from species-specific differences. Although Ca\textsuperscript{2+} oscillations have been observed in airway epithelial cells from rabbit, cow (21), mouse (Evans, unpublished observations), and sheep (37), there has been no report of Ca\textsuperscript{2+} oscillations in human airway cells except for serous gland cells (25). In view of the widespread occurrence of Ca\textsuperscript{2+} oscillations, it would be surprising to find that human cells fail to demonstrate Ca\textsuperscript{2+} oscillations.

Differences in the signaling components and the Ca\textsuperscript{2+} response of cells can also arise from culturing conditions such as variations in the composition of the matrix and medium, variations in the Ca\textsuperscript{2+}-signaling phenotype between normal and transformed cell lines, and the applied agonist concentrations. In addition, [Ca\textsuperscript{2+}]i measurements from populations of cells rather than from individual cells would fail to reveal heterogeneous Ca\textsuperscript{2+} oscillations such as those reported here. These reasons may also account for why the observation of Ca\textsuperscript{2+} oscillations in airway epithelial cells has varied among investigators.

If the steady-state [IP\textsubscript{3}]i is controlled by the degree of receptor activation, as it is in other cell types (4), then the four basic Ca\textsuperscript{2+} responses observed in airway epithelial cells reflect the changes in [IP\textsubscript{3}]i, a hypothesis that is consistent with the mathematical modeling of Ca\textsuperscript{2+} oscillations (43). When the [ATP]o was relatively low (relative to the intrinsic sensitivity of the cell to ATP\textsubscript{o}), reflecting relatively low receptor activation and a low steady-state [IP\textsubscript{3}]i, the cell was unable to support Ca\textsuperscript{2+} oscillations and responded to ATP\textsubscript{o} with a single [Ca\textsuperscript{2+}]i increase. In the [ATP]o range that yielded Ca\textsuperscript{2+} oscillations, the Ca\textsuperscript{2+} oscillation frequency increased with increased [ATP]o, an observation common to many cell types stimulated by a wide variety of agonists such as vasopressin (33), phenylephrine (34), acetylcholine (29), and ATP (8) and possibly reflected increased [IP\textsubscript{3}]i.

Spatial characteristics of Ca\textsuperscript{2+} oscillations. The pattern of ATP-induced Ca\textsuperscript{2+} oscillations was intrinsic to each cell, and Ca\textsuperscript{2+} oscillations within one cell failed to influence the [Ca\textsuperscript{2+}]i activity of adjoining cells in a
manner similar to the asynchronous ATP-induced Ca\(^{2+}\) oscillations that have been observed in bile duct cells (29) and MDCK cells (35) and are consistent with the behavior of spontaneous Ca\(^{2+}\) oscillations in airway epithelial cells (6). After a nearly synchronous increase in [Ca\(^{2+}\)]i, in the cells in response to the addition of ATP\(_{o}\), each cell displayed a unique Ca\(^{2+}\) response. In contrast, ATP-induced Ca\(^{2+}\) oscillations initiate intercellular Ca\(^{2+}\) waves in cultures of chondrocytes (8), and a variety of agonists generate Ca\(^{2+}\) oscillations that spread as intercellular Ca\(^{2+}\) waves in liver tissue (28, 33) and hepatocytes (48). Interestingly, in MDCK cells, bradykinin and thrombin, but not ATP\(_{o}\), stimulate synchronized Ca\(^{2+}\) oscillations, suggesting that the synchrony of Ca\(^{2+}\) oscillations in adjoining cells may be a function of the agonist (35). The synchrony of Ca\(^{2+}\) oscillations in MDCK cells and the spreading of intercellular Ca\(^{2+}\) waves from Ca\(^{2+}\) oscillations in hepatocytes and salivary glands, however, are dependent on gap junction communication. Disruption of communication by octanol (35) or α-glycyrrhetinic acid (48) leads to asynchronous Ca\(^{2+}\) oscillations or the failure of Ca\(^{2+}\) oscillations to initiate intercellular Ca\(^{2+}\) waves without affecting Ca\(^{2+}\) oscillations themselves, suggesting the gap junction communication of a signaling molecule between cells to maintain synchrony.

Intercellular communication during Ca\(^{2+}\) oscillations. In airway epithelial cells, mechanical stimulation of one cell results in propagated increases in [Ca\(^{2+}\)]i to neighboring cells or an intercellular Ca\(^{2+}\) wave. Passage of an intercellular Ca\(^{2+}\) wave in airway epithelial cells is mediated by the gap junction diffusion of IP\(_3\), generated in the stimulated cell (6). Consequently, the passage of an intercellular Ca\(^{2+}\) wave through a field of ATP-stimulated cells, some of which were displaying Ca\(^{2+}\) oscillations, suggests that the inability of the Ca\(^{2+}\)-oscillatory behavior of one cell to influence a neighboring cell is not due to the disruption of gap junction communication between cells. This inability instead demonstrates that Ca\(^{2+}\) alone is insufficient as an intercellular messenger to communicate Ca\(^{2+}\) changes to neighboring airway epithelial cells even when the IP\(_3\)Rs of the neighboring cells are sensitized to IP\(_3\), although Ca\(^{2+}\) may act as the messenger in other cell types (28, 33, 48). Indeed, the magnitude of the Ca\(^{2+}\) increase associated with the Ca\(^{2+}\) oscillations in the cells is not different from the magnitude of the [Ca\(^{2+}\)]i change associated with the passage of the intercellular Ca\(^{2+}\) wave, thus demonstrating that the [Ca\(^{2+}\)]i increase associated with the wave cannot account for Ca\(^{2+}\) wave transmission.

Although it has been reported that mechanical stimulation of airway epithelial cells results in the release of ATP (14), we do not believe that the intercellular propagation of Ca\(^{2+}\) waves described above relies on this mechanism. We repeatedly failed to observe Ca\(^{2+}\) oscillations in cells after the passage of a mechanically stimulated intercellular Ca\(^{2+}\) wave, which we would expect if Ca\(^{2+}\) waves were dependent on ATP\(_{o}\). Also, although the Ca\(^{2+}\) response in airway epithelial cells is blocked by suramin, a P2-receptor antagonist, the propagation of mechanically stimulated intercellular Ca\(^{2+}\) waves is not (17). Moreover, fluid flow over cells during mechanical stimulation fails to bias the direction of spread of the subsequent intercellular Ca\(^{2+}\) waves (17).

Associated with the passage of the intercellular Ca\(^{2+}\) wave through ATP-induced Ca\(^{2+}\)-oscillating cells was a transient increase in the oscillation frequency and the initiation of Ca\(^{2+}\) oscillations at a higher [Ca\(^{2+}\)]i. As discussed above, this observation is consistent with a transient increase in [IP\(_3\)]i over the steady-state [IP\(_3\)]i, which would be expected because IP\(_3\) diffuses outward from the stimulated cell ahead of the Ca\(^{2+}\) wave and then is metabolized and diffused into the surrounding cells. Although IP\(_3\) can apparently traverse the gap junctions between neighboring cells stimulated with ATP\(_{o}\), we suggest that no IP\(_3\) gradient is established between ATP-stimulated cells, so no intercellular Ca\(^{2+}\) waves are initiated from any cell.

In summary, two separate Ca\(^{2+}\) signals can occur in airway epithelial cells: Ca\(^{2+}\) oscillations that are intrinsic and confined to an individual cell and intercellular Ca\(^{2+}\) waves that are generated at a distance and encompass a number of cells. We show that the two Ca\(^{2+}\) signals can occur simultaneously within cells and suggest that Ca\(^{2+}\) oscillations may serve to regulate individual cell functions, whereas intercellular Ca\(^{2+}\) waves coordinate cooperative cell activity.

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