Intracellular calcium oscillations induced by ATP in airway epithelial cells

JOHN H. EVANS 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 (ATPₒ) stimulates a transient increase in intracellular Ca²⁺ concentration that is followed by periodic increases in intracellular Ca²⁺ concentration (Ca²⁺ oscillations). The characteristics and mechanism of these ATP-induced Ca²⁺ responses were studied in primary cultures of rabbit tracheal epithelial cells with digital video microscopy and the Ca²⁺-indicator dye fura 2. The continual presence of ATPₒ at concentrations of 0.1–100 µM stimulated Ca²⁺ oscillations that persisted for 20 min. The frequency of the Ca²⁺ oscillations was found to be dependent on both ATPₒ concentration and intrinsic sensitivity of each cell to ATPₒ. Cells exhibited similar Ca²⁺ oscillations to extracellular UTP (UTPₒ), but the oscillations typically occurred at lower UTPₒ concentrations. The ATP-induced Ca²⁺ oscillations were abolished by the phospholipase C inhibitor U-73122 and by the endoplasmic reticulum Ca²⁺-pump inhibitor thapsigargin but were maintained in Ca²⁺-free medium. These results are consistent with the hypothesis that in airway epithelial cells ATPₒ and UTPₒ act via P₂U purinoceptors to stimulate Ca²⁺ oscillations by the continuous production of inositol 1,4,5-trisphosphate and the oscillatory release of Ca²⁺ from internal stores. ATP-induced Ca²⁺ oscillations of adjacent individual cells occurred independently of each other. In contrast, a mechanically induced intercellular Ca²⁺ wave propagated through a field of Ca²⁺-oscillating cells. Thus Ca²⁺ oscillations and propagating Ca²⁺ waves are two fundamental modes of Ca²⁺ signaling that exist and operate simultaneously in airway epithelial cells.


Oscillatory changes in intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ), 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⁺]ᵢ. 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⁺]ᵢ. Once the [Ca²⁺]ᵢ falls to a permissive level, repetitive cycles of [Ca²⁺]ᵢ increase and decline are maintained by an elevated intracellular IP₃ concentration ([IP₃]ᵢ) (13, 15). Thus Ca²⁺ oscillations are dependent on the action of both [Ca²⁺]ᵢ and [IP₃]ᵢ on the IP₃R. Experimental data show that the frequency of Ca²⁺ oscillations are dependent on [IP₃]ᵢ, and, indirectly, agonist concentration (15). Mathematical modeling of Ca²⁺ oscillations as a function of [IP₃]ᵢ and [Ca²⁺]ᵢ agrees with the experimental findings (43).

Extracellular ATP (ATPₒ) serves as an agonist in many organs and tissues (12) and induces an initial transient increase in [Ca²⁺]ᵢ, 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, ATPₒ mobilizes [Ca²⁺]ᵢ 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 (UTPₒ) as well as to ATPₒ (19, 26). ATPₒ and UTPₒ 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 ATPₒ or UTPₒ 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⁺]ᵢ 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 synchrony of Ca²⁺ oscillations in the above systems invariably relies on gap junction communication between contacting cells, and the synchrony 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 synchrony 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 through the gap junctions to initiate intercellular Ca²⁺ waves or whether the intercellular messenger em-
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+]i (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+]i 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 ([ATP]o), were mediated by G protein-coupled receptors involving an IP3 signaling pathway, and were ATPo concentration ([ATP]o), displayed periodic Ca2+ waves under a number of experimental conditions.

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+]i increase varied greatly from cell to cell, but in general, it increased with increasing [ATP]o. After the initial [Ca2+]i increase induced by ATPo 7–36% of all the cells, depending on the [ATP]o, 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+]i transient and consisted of a sharp increase in [Ca2+]i followed by a slower [Ca2+]i decline; however, the [Ca2+]i 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+]i 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

MATERIALS AND METHODS

The techniques for culturing airway epithelial cells, mechanically stimulating individual cells, and the measurement of [Ca2+]i 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 tracheae 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 ~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+]i. Cells were loaded with fura 2 by incubation at 37°C for 1 h in 5 µM fura 2-AM (Calbiochem) in Ca2+-free Hanks’ buffered salt solution without phenol red (HBSS; Gibco BRL) and additionally buffered with 25 mM HEPES (HBSS, 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 HBSS. 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+]i were calculated by single-wavelength recordings referenced to ratiometric measurements (23). Initial [Ca2+]i reference images were based on 10 frames recorded at 340 and 380 nm. Changes in [Ca2+]i 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+]i 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. For plots of [Ca2+]i versus time, single points encompassing an area of 2.1 × 2.3 µm were selected from the cells of interest, and [Ca2+]i was calculated only at those points. Time-lapse recordings were made at 1 images/s (each frame recorded at 30 frames/s).

Drug application. ATPo and UTPo (Sigma) were dissolved in distilled H2O at 10 mM and stored in aliquots at −20°C. Desired final concentrations were made by dilution of the stock in HBSS or Ca2+/-Mg2+-free DPBS. Thapsigargin, U-73122, and U-73343 (BIOMOL) were dissolved in dimethyl sulfoxide (DMSO; 1.5, and 7.5 mM, respectively), divided into aliquots, and stored at −20°C. Final concentrations were made by dilution in HBSS. Controls for thapsigargin experiments were performed in 0.1% DMSO. Two hundred microliters of the required experimental solution were exchanged for the 200 µl of HBSS in the cell chamber for each experiment. Between trials, the cells were washed with >3 ml (>15 volumes) of HBSS. In all experiments, the cells were allowed to recover for >15 min between trials or between control and experimental conditions.

Mechanical stimulation. Mechanical stimulation of a single cell was performed by brief displacement of the apical surface of the cell membrane with a glass micropipette (~1-µm tip diameter) for 100 ms. The magnitude and duration of the membrane displacement was controlled by applying a voltage pulse with a Grass stimulator to a piezoelectric crystal to which the micropipette was attached (40).
Ca^2+ pools or IP_3Rs. 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+}]_i. 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...

Fig. 1. Response of individual airway epithelial cells to extracellular ATP (ATP_o). Representative traces of intracellular Ca^{2+} concentration ([Ca^{2+}]_i) changes with respect to time of 3 different cells exposed to increasing ATP_o concentration ([ATP]_o; 0.1–100 µM) are shown. Sensitivities of cells were determined by minimum [ATP]_o value required to elicit multiple Ca^{2+} oscillations. A: high ATP-sensitive cells displayed multiple Ca^{2+} oscillations at ~0.5 µM ATP. B: intermediate ATP-sensitive cells displayed Ca^{2+} oscillations at ~5 µM ATP. C: low ATP-sensitive cells displayed Ca^{2+} oscillations at ~50 µM ATP. Characteristics of Ca^{2+} oscillation responses to ATP_o were similar between each ATP sensitivity category: frequency of Ca^{2+} oscillations increased with increased [ATP]_o until, at high relative [ATP]_o values, Ca^{2+} oscillations ceased and [Ca^{2+}]_i remained elevated. Traces are representative of data sets of 265 cells analyzed from 5 independent experiments.
increase. However, the initial \([\text{Ca}^{2+}]_i\) transients were virtually identical for both agonists. In a third cell (Fig. 3C), \(\text{UTP}_o\) elicited a higher frequency of \(\text{Ca}^{2+}\) oscillations compared with that elicited by \(\text{ATP}_o\).

Of cells displaying an \([\text{Ca}^{2+}]_i\) increase in response to \(0.1 \mu M \text{UTP}\), 91% of these cells exhibited an \([\text{Ca}^{2+}]_i\) increase in response to \(0.1 \mu M \text{ATP}\) (n = 229 cells, 4 experiments; Fig. 4A). Less than 2% of cells exhibited an \([\text{Ca}^{2+}]_i\) increase in response to \(\text{ATP}_o\) but not to \(\text{UTP}_o\). Also, \(0.1 \mu M \text{UTP}\) initiated \(\text{Ca}^{2+}\) oscillations in 59% of cells compared with 40% with \(0.1 \mu M \text{ATP}\) (Fig. 4A), and the cells stimulated with \(\text{UTP}_o\) showed a greater number of oscillations than those stimulated with \(\text{ATP}_o\) (Fig. 4B). These responses to \(\text{ATP}_o\) or \(\text{UTP}_o\) were independent of the order of agonist challenge.
Ca²⁺ oscillations and PLC activity.

The stimulation of [Ca²⁺]ᵢ increase by ATP₀ and UTP₀ via P₂U receptors is believed to involve the release of Ca²⁺ from IP₃-sensitive Ca²⁺ stores after the activation of PLC and the production of IP₃ (12). In support of this hypothesis, Hansen et al. (16) found that neither ryanodine nor caffeine increases [Ca²⁺]ᵢ in airway epithelial cells and suggested that the internal Ca²⁺ release does not appear to involve a ryanodine receptor-mediated Ca²⁺-induced Ca²⁺ release. The involvement of PLC in the response of epithelial cells to ATP₀ is supported by the finding that an increase in [Ca²⁺]ᵢ was abolished by the aminosteroid U-73122, a PLC inhibitor, whereas its inactive analog U-73343 had no effect (17). To determine whether PLC activation was part of the mechanism of ATP-induced Ca²⁺ oscillations, we investigated the effect of U-73122 on ATP-induced Ca²⁺ oscillations.

Incubation of cells for 20 min in 10 µM U-73122 before application of 5 µM ATP completely abolished the initial [Ca²⁺]ᵢ transient as well as the subsequent Ca²⁺ oscillations in the cells (data not shown). A 20-min incubation in 10 µM U-73343 had no effect on the initial [Ca²⁺]ᵢ response or subsequent Ca²⁺ oscillations (data not shown). Because it is possible that Ca²⁺ oscillations require an initial PLC-dependent [Ca²⁺]ᵢ increase but do not require continual activity of PLC, we added 10 µM U-73122 to cells exhibiting ongoing ATP-induced Ca²⁺ oscillations. ATP-induced Ca²⁺ oscillations quickly ceased on addition of U-73122 (Fig. 5A). The addition of U-73343 had no effect on ongoing Ca²⁺ oscillations (n = 83 cells, 3 experiments; Fig. 5B).

Ca²⁺ oscillations and the release of internal Ca²⁺ stores. IP₃-mediated Ca²⁺ oscillations typically are dependent on Ca²⁺ release from internal stores and are independent of the extracellular Ca²⁺ concentration ([Ca²⁺]₀). To investigate the dependence of ATP-induced Ca²⁺ oscillations on [Ca²⁺]₀, cells were stimulated with 5 µM ATP in Ca²⁺/Mg²⁺-free DPBS. Because extracellular unbound EGTA has been shown to interfere with Ca²⁺ release from intracellular stores in airway epithelial cells (18) and because the Ca²⁺ response to histamine in airway cells was essentially the same in nominally Ca²⁺-free medium, medium containing 1 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetie acid, and medium with an [Ca²⁺]₀ of ~7 nM (18), we decided to use a nominally Ca²⁺-free medium for these experiments. Ca²⁺ oscillations induced by ATP₀ occurred in the absence of extracellular Ca²⁺ (n = 33 cells, 3 experiments; Fig. 6A). Treatment with Ca²⁺/Mg²⁺-free DPBS alone failed to induce Ca²⁺ oscillations (Fig. 6B).

To investigate further the dependence of Ca²⁺ oscillations on internal Ca²⁺ stores, we used the Ca²⁺-ATPase inhibitor thapsigargin. Prolonged incubation (20 min) of cells with 1 µM thapsigargin elevated the baseline 

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**Fig. 4.** Summary of differential responses to ATP₀ and UTP₀. A: of the cells which exhibited at least 1 [Ca²⁺]ᵢ increase in response to 0.1 µM UTP, 91% also responded to 0.1 µM ATP. Only 4 of 229 cells from 4 experiments that responded to ATP₀ failed to respond to UTP₀. Of the responding cells, 59% of cells exhibited oscillations in UTP₀, whereas only 40% exhibited oscillations in ATP₀. B: average no. of oscillations/cell induced by UTP₀ was higher than that induced by ATP₀ in the 5-min recording period.

**Fig. 5.** Effect of phospholipase C inhibitor U-73122 and an inactive analog, U-73343, on [Ca²⁺]ᵢ changes induced by ATP₀. A: U-73122 was added to cells 2 min after exposure to 5 µM ATP. Trace shows that ATP₀ induced a characteristic Ca²⁺ oscillation pattern and that Ca²⁺ oscillations ceased after addition of U-73122. B: similar experiment shows that U-73343 had no effect on Ca²⁺ oscillations (n = 83 cells from 3 experiments).
[Ca\textsuperscript{2+}] in the cells and abolished the Ca\textsuperscript{2+} response of the cells to 5 µM ATP (data not shown). Again, Ca\textsuperscript{2+} oscillations may be dependent on an initial Ca\textsuperscript{2+} increase but independent of internal Ca\textsuperscript{2+} pools. To investigate this possibility, cells were exposed for a short time (1 min) to thapsigargin before stimulation with ATP\textsubscript{o}. This short-term exposure to thapsigargin stimulated a small rise in [Ca\textsuperscript{2+}] but allowed a large ATP-induced [Ca\textsuperscript{2+}] increase (n = 91 cells, 4 experiments; Fig. 7A). Baseline [Ca\textsuperscript{2+}] levels remained elevated in these cells, and the cells failed to exhibit Ca\textsuperscript{2+} oscillations. An identical treatment of cells with DMSO, the vehicle for thapsigargin, resulted in a normal Ca\textsuperscript{2+} response to ATP\textsubscript{o} treatment (Fig. 7B).

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

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

Intercellular Ca\(^{2+}\) signaling. In a previous study (6), it has been demonstrated that in response to mechanical stimulation, airway epithelial cells propagated increases in [Ca\(^{2+}\)]~i~ similar to those associated with Ca\(^{2+}\) oscillations to adjacent cells as intercellular Ca\(^{2+}\) waves. The mechanism responsible for the intercellular waves was proposed to be the diffusive spread of IP\(_3\) rather than of Ca\(^{2+}\) between adjacent cells via gap junctions. Consequently, the asynchronous nature and failure of ATP-induced Ca\(^{2+}\) oscillations to influence the 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 Ca\(^{2+}\) oscillations to propagate intercellular Ca\(^{2+}\) waves was tested.

Figure 10 shows pseudocolor images of the change in [Ca\(^{2+}\)]~i~ in confluent cells that have been exposed to 5 µM ATP and have been subsequently mechanically stimulated. In response to ATP\(_o\), many cells exhibit 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 Ca\(^{2+}\) responses in adjacent cells. After mechanical stimulation of a single cell (Fig. 10D, arrow), an intercellular Ca\(^{2+}\) wave spread through Ca\(^{2+}\)-oscillating and nonoscillating cells (Fig. 10, E and F). After passage of the intercellular Ca\(^{2+}\) wave (Fig. 10, G–I), many cells (**) resumed Ca\(^{2+}\) oscillations. The traces in Fig. 11 show typical [Ca\(^{2+}\)]~i~ responses of several adjacent cells after application of ATP\(_o\); the
Ca\textsuperscript{2+} oscillations in cell B rapidly increased and subsequently decreased to a higher baseline [Ca\textsuperscript{2+}]. In cell C, the [Ca\textsuperscript{2+}] increased after a ~3-s delay to a higher amplitude than the preceding oscillatory [Ca\textsuperscript{2+}] increases. Interestingly, the next two Ca\textsuperscript{2+} oscillations initiated after a shorter period and at a higher baseline [Ca\textsuperscript{2+}], than did the Ca\textsuperscript{2+} oscillations preceding the intracellular wave. In cell D, after a ~5-s delay, the [Ca\textsuperscript{2+}] increase in the intercellular Ca\textsuperscript{2+} wave was higher than the initial [Ca\textsuperscript{2+}] increase; however, it failed to reinitiate Ca\textsuperscript{2+} oscillations.

**DISCUSSION**

In this study, we demonstrated by digital imaging fluorescence microscopy that airway epithelial cells exhibit Ca\textsuperscript{2+} oscillations in response to ATP\textsubscript{o}. Although Ca\textsuperscript{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\textsuperscript{2+} oscillations, the intercellular heterogeneity of ATP\textsubscript{o} responsiveness, and the intercellular signaling associated with ATP-induced Ca\textsuperscript{2+} oscillations.

Signalng pathway involved in the generation of Ca\textsuperscript{2+} oscillations. Airway epithelial cells exhibit a suramin-sensitive [Ca\textsuperscript{2+}] response to ATP\textsubscript{o} (16), which suggests that P2 purinergic receptors are involved in the [Ca\textsuperscript{2+}] 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\textsuperscript{2+}]\textsubscript{i} for activity, was not active at high (1.5 mM) [Ca\textsuperscript{2+}]\textsubscript{i}, and was not activated by UTP\textsubscript{o} (22). Because our experiments were conducted in ~1.3 mM [Ca\textsuperscript{2+}]\textsubscript{i}, we believe that P2X-receptor activity did not contribute to the [Ca\textsuperscript{2+}] increase observed.

U-73122, when added to cells experiencing ATP-induced Ca\textsuperscript{2+} oscillations and was not activated by U-73122, when added to cells experiencing ATP-induced Ca\textsuperscript{2+} oscillations.

The aminosteroid U-73122, a PLC antagonist, prevented an initial [Ca\textsuperscript{2+}] increase in response to ATP\textsubscript{o} consistent with an earlier report suggesting that the Ca\textsuperscript{2+} response to ATP\textsubscript{o} was mediated by a PLC/IP\textsubscript{3} pathway (17). U-73122, when added to cells experiencing ATP-induced Ca\textsuperscript{2+} oscillations, resulted in the immediate cessation of Ca\textsuperscript{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 ATPo 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}] 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}] 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 ATPo 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 ATPo 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+}]i during thapsigargin treatment; however, when ATPo was applied, the cells responded with a large initial [Ca\textsuperscript{2+}]i 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+}]i was increased until the Ca\textsuperscript{2+} oscillations ceased at an
Ca\textsuperscript{2+} OSCILLATIONS IN AIRWAY CELLS

A* B C D

Fig. 11. Initiation of an intercellular Ca\textsuperscript{2+} wave through cells experiencing ATP-induced Ca\textsuperscript{2+} oscillations. Outlines (top) and Ca\textsuperscript{2+} traces (bottom) of 4 cells treated with 5 \mu M ATP are shown. ATP\textsubscript{o} was applied to cells (line A) and elicited characteristic Ca\textsuperscript{2+} oscillations in the 4 cells. After 120 s, cell A was mechanically stimulated with an micropipette (line S), which resulted in an immediate increase in [Ca\textsuperscript{2+}]\textsubscript{i} of cell A and generation of an intercellular Ca\textsuperscript{2+} wave. Sequential increases in [Ca\textsuperscript{2+}]\textsubscript{i} associated with intercellular Ca\textsuperscript{2+} wave were observed in cells B-D, with an increasing time delay. [Ca\textsuperscript{2+}]\textsubscript{i} increase in cell B was transient and resulted in a slightly elevated baseline [Ca\textsuperscript{2+}]\textsubscript{i}. In cell C, [Ca\textsuperscript{2+}]\textsubscript{i} increase was followed first by a single Ca\textsuperscript{2+} oscillation that had a shorter period and initiated at a higher [Ca\textsuperscript{2+}]\textsubscript{i} than the previous Ca\textsuperscript{2+} oscillations and then by resumption of Ca\textsuperscript{2+} oscillations similar to those occurring before Ca\textsuperscript{2+} wave. In cell D, [Ca\textsuperscript{2+}]\textsubscript{i} increase was transient and failed to reinitiate Ca\textsuperscript{2+} oscillations.

Elevated [Ca\textsuperscript{2+}]\textsubscript{i}. A dependence on thapsigargin-sensitive internal Ca\textsuperscript{2+} pools is a similar requirement for the ATP-induced Ca\textsuperscript{2+} oscillations of chondrocytes (8) and bile duct epithelial cells (29).

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+}]\textsubscript{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]\textsubscript{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+}] values, a correlation between ATP sensitivity and resting [Ca\textsuperscript{2+}] 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+}]\textsubscript{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}] 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}], a hypothesis that is consistent with the mathematical modeling of Ca\textsuperscript{2+} oscillations (43). When the [ATP]\textsubscript{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}], the cell was unable to support Ca\textsuperscript{2+} oscillations and responded to ATP\textsubscript{o} with a single [Ca\textsuperscript{2+}] increase. In the [ATP]\textsubscript{o} range that yielded Ca\textsuperscript{2+} oscillations, the Ca\textsuperscript{2+} oscillation frequency increased with increased [ATP]\textsubscript{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}]. In some experiments, the first few Ca\textsuperscript{2+} oscillations had shorter periods and initiated at a higher [Ca\textsuperscript{2+}]\textsubscript{i} than the subsequent Ca\textsuperscript{2+} oscillations.

Exhibition of preceding higher-frequency Ca\textsuperscript{2+} oscillations initiating at higher [Ca\textsuperscript{2+}]\textsubscript{i} values is a feature common in cells to many agonists including ATP (8, 29, 46). At relatively high [ATP]\textsubscript{o} values, when the steady-state [IP\textsubscript{3}] would be high, the cells responded with a sustained increase in [Ca\textsuperscript{2+}] and no Ca\textsuperscript{2+} oscillations.

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+}] 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\(^{2+}\)], in the cells in response to the addition of ATP, 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, 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 \(\alpha\)-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\(^{2+}\)] 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\(^{2+}\)], change associated with the passage of the intercellular Ca\(^{2+}\) wave, thus demonstrating that the [Ca\(^{2+}\)] 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. 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\(^{2+}\)]. As discussed above, this observation is consistent with a transient increase in [IP\(_3\)] over the steady-state [IP\(_3\)], 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, 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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