Cell to Cell Communication in Response to Mechanical Stress via Bilateral Release of ATP and UTP in Polarized Epithelia

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Abstract. Airway epithelia are positioned at the interface between the body and the environment, and generate complex signaling responses to inhaled toxins and other stresses. Luminal mechanical stimulation of airway epithelial cells produces a propagating wave of elevated intracellular Ca$^{2+}$ that coordinates components of the integrated epithelial stress response. In polarized airway epithelia, this response has been attributed to IP$_3$ permeation through gap junctions. Using a combination of approaches, including enzymes that destroy extracellular nucleotides, purinergic receptor desensitization, and airway cells deficient in purinoceptors, we demonstrated that Ca$^{2+}$ waves induced by luminal mechanical stimulation in polarized airway epithelia were initiated by the release of the 5′ nucleotides, ATP and UTP, across both apical and basolateral membranes. The nucleotides released into the extracellular compartment interacted with purinoceptors at both membranes to trigger Ca$^{2+}$ mobilization. Physiologically, apical membrane nucleotide-release coordinates airway mucociliary clearance responses (mucin and salt, water secretion, increased ciliary beat frequency), whereas basal membrane release constitutes a paracrine mechanism by which mechanical stresses signal adjacent cells not only within the epithelium, but other cell types (nerves, inflammatory cells) in the submucosa. Nucleotide-release ipsilateral and contralateral to the surface stimulated constitutes a unique mechanism by which epithelia coordinate local and distant airway defense responses to mechanical stimuli.

Key words: intercellular Ca$^{2+}$ wave • nucleotide-release • airway epithelium • mechanical stimulus • P$_2$-receptors

Introduction

Airway epithelia in vivo are exposed to a spectrum of chemical and mechanical stresses. As a part of normal airways defense, epithelia must mount coordinated responses to prevent damage/toxicity. Because the airways epithelia are not innervated, it has not been clear how airway epithelial cells respond in a coordinated fashion to perform these defense-related activities.

Because many of the epithelial defense functions are regulated by changes in intracellular Ca$^{2+}$ levels, it has been attractive to focus on regulation of Ca$^{2+}$ signaling in cells exposed to stress. Indeed, mechanically induced intercellular Ca$^{2+}$ waves have been described in several cell types, including epithelial cells studied under nonpolarized conditions (e.g., hepatocytes, mammary cells) (Enomoto et al., 1994; Schlosser et al., 1996; Frame and de Feijter, 1997), as well as a variety of nonepithelial cells (e.g., basophilic leukemia cells, glial cells, and insulin-secreting cells; Osipchuk and Cahalan, 1992; Cao et al., 1997; Newman and Zahs, 1997). It has been proposed that the propagation of Ca$^{2+}$ waves in these cells is mediated by extracellular nucleotides. In contrast, a distinct mechanism mediating intercellular Ca$^{2+}$ waves has been proposed for polarized epithelial cells (Boitano et al., 1992; Hansen et al., 1993; Sneyd et al., 1995; Sanderson, 1996; Dirksen 1998; Felix et al., 1998). In this model, IP$_3$ generated in the mechanically stimulated cell permeates to adjacent cells via gap junctions, resulting in release of Ca$^{2+}$ from internal stores and propagation of a Ca$^{2+}$ wave.

A number of observations have raised the possibility that extracellular nucleotide signaling may also be pertinent to polarized airway epithelia. For example, polarized airway epithelial cells functionally express P2Y-purinoceptors that are activated by both adenine and uridine nucleotides (Brown et al., 1991; Mason et al., 1991; Paradiso et al., 1995; Hwang et al., 1996; Lazarowski et al., 1997a; Cressman et al., 1998, 1999; Homolya et al., 1999). Further...
thermore, mechanical stimulation induces release of cellular ATP from airway epithelial cells (Felix et al., 1996; Grygorczyk and Hanrahan, 1997; Watt et al., 1998). More recently, we reported that mechanical stress also elicits UTP-release from airway epithelial cells (Lazarowski et al., 1997b).

Therefore, we tested the hypothesis that extracellular ATP and UTP mediate mechanically induced intracellular $Ca^{2+}$ waves in airway epithelia. We generated unique airway epithelial model systems, including nasal epithelial cell lines from wild-type and P2Y$_6$–8 h.

To establish (x$^2$/y$^2$), we used the fluorescent indicator, Fura-2, and the intracellular $Ca^{2+}$ concentration of reagents.

Materials and Methods

Airway Epithelial Cell Cultures

Immortalized nasal epithelial cells obtained from normal and P2Y$_6$–R (−/−) mice (Homolya et al., 1999) were maintained on 24-mm Transwell Col filters (pore diameter 0.45 µm; Corning Costar) in Ham’s F12-based medium containing 10 µg/ml insulin, 5 µg/ml transferrin, 1 µM hydrocortisone, 30 nM triiodothyronine, 25 ng/ml epidermal growth factor, 3.75 µg/ml endothelial cell growth substance, 0.8 mM Ca$_{2+}$ (total), and an equal amount of 3T3 fibroblast-conditioned DME containing 2% FBS. For imaging studies, the cells were seeded at 5 × 10$^4$ cells/cm$^2$ density on Transwell Col filters previously coated with 0.03 mg/ml Vitrogen. Studies were carried out with confluent mouse cultures 5–7 d after seeding. Confluence was assessed visually and by measuring transepithelial electrical resistance (Rt) with an EVOM (WPI). Mean values were determined by using 1 µM Fura-2 free acid and solutions of known Ca$_{2+}$ concentration.

Viability Tests

As one approach, the viability of the cells was tested by the response to carbachol (1 mM) added to the basolateral bath after each protocol initiated by mechanical stimulation. As a second approach to test the viability, in selected experiments the cultures were incubated with ethidium-homodimer (10 mM), and red fluorescence (620 ± 15 nm) acquired after 520-nm excitation. The sensitivity of this viability assay was tested by exposing all the cells in the culture to digitonin (80 µM) in the continuous presence of the dye.

Reagents

Hormones for cell culture were purchased from Collaborative Research, Inc., with the exception of triiodothyronine, which was from Sigma-Aldrich; vitrogen, which was from Cohesion Technologies; and other cell culture materials were purchased from Life Technologies, Inc. Molecular biology grade ATP and UTP were purchased from Amersham Pharmacia Biotech. Hexokinase, UDP, ADP, and adenosine were from Roche Molecular Biochemicals. Digitonin was obtained from RBI, Fura-2 AM, Fura-2 pentapotassium salt, ethidium-homodimer, and Ca$_{2+}$ calibration buffers were purchased from Molecular Probes. All other chemicals including ipryase with an exceptionally low ATPase/ADPase ratio (grade V) were purchased from Sigma-Aldrich.

To remove triphosphate contamination from diphosphate nucleotides, 1 mM stock solutions of UDP and ADP were pretreated with 10 U/ml hexokinase for 30 min at 37°C in the presence of 5 mM glucose (Lazarowski et al., 1997a).

Data Analysis

The background corrected ratio images (340/380) calibrated by the formula of Grynkiewicz et al. (1985) were replayed. The stimulated cell was identified and regions of interest (70 µm apart) were diagonally selected (see below). For concentration effect curves, full field photometry was applied, and differences between the peak and basal Ca$_{2+}$ concentration were plotted. All the data are presented as mean ± SEM. For comparisons, the mean values were analyzed by unpaired t tests.

Results

Quantitation of Mechanically Induced Ca$^{2+}$ Waves

Polarized cultures of normal mouse nasal cells were loaded with the fluorescent indicator, Fura-2, and the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) monitored by video imaging. The apical membrane, i.e., luminal surface, of a single cell within a confluent culture was briefly displaced with a micropipette, which resulted in a radially propagating Ca$^{2+}$ wave (Fig. 1a). The viability of each cell after mechanical stimulation was demonstrated by impermeability to ethidium-homodimer (Fig. 1b), the persistence of receptor-mediated Ca$^{2+}$ responses elicited by the muscarinic recep-
tor agonist carbachol (see Fig. 1, a and c), and the absence of Fura-2 leakage.

To quantitate wave propagation, \([\text{Ca}^{2+}]_i\) was measured in a circular region of interest (ROI; 27.5-μm diameter) at the point of stimulation (ROI_0), and at distances 70- and 140-μm (≈4 and ≈8 cells) from the stimulated cell (ROI_70 and ROI_140, respectively; see bright field image in Fig. 1 c, left). The magnitude of the \(\text{Ca}^{2+}\) response was greatest in the stimulated cell and declined as a function of time and distance from this cell (middle). The \(\text{Ca}^{2+}\) level in ROI_0 typically reached its peak eight seconds after the stimulus, whereas peak responses were recorded at 12 and 16 s for ROI_70 and ROI_140, respectively (n = 20). The \([\text{Ca}^{2+}]_i\) at times of peak responses were used for calculating and plotting mean maximum \(\text{Ca}^{2+}\) responses (right).

**Ca\(^{2+}\) Wave Propagation Across Discontinuous Airway Epithelia: Sensitivity to Apyrase**

We used two approaches to test whether the intercellular \(\text{Ca}^{2+}\) waves required cell–cell contact. First, we generated short-term cultures (overnight) in which the nonpolarized epithelial cells were not confluent, i.e., the cells grew in separated islands (Fig. 2 a). Mechanical stimulation of a single cell induced a \(\text{Ca}^{2+}\) wave (top row) that was transmitted to cells not in physical contact with the stimulated cell. Apyrase (10 U/ml, grade V), an enzyme that rapidly hydrolyses 5‘-nucleotide-triphosphates to monophosphates, prevented the propagation of the \(\text{Ca}^{2+}\) signal to the adjacent cells (bottom row), suggesting that the mediator of the \(\text{Ca}^{2+}\) wave was a 5‘-nucleotide. The carbachol-induced \(\text{Ca}^{2+}\) response was not affected by apyrase (data not shown).

The second approach tested whether the concept of extracellular nucleotide release was also pertinent after the airway epithelial cells had fully polarized (Fig. 2 b). A small number of cells were physically removed from a region of confluent polarized culture to produce a linear gap and a single cell was stimulated on one side of this discontinuity. The propagating \(\text{Ca}^{2+}\) wave skipped over the gap (Fig. 2 b, top row), indicating involvement of a released,
diffusible substance(s). Note that the magnitude of the responses was not different at equal distances from the stimulated cell (ROI 70 and 140), irrespective of the presence of a gap. Apyrase abolished the transmission of the Ca^2+ spread (Fig. 2b, bottom row).

Evidence that Apyrase Sensitive Mediators (Nucleotide) Are Released from both Apical and Basolateral Surfaces in Response to Mechanical Stimulation

In polarized airway epithelial cell cultures, apyrase added selectively to the apical bath alone attenuated, but did not block the mechanically induced intercellular Ca^2+ wave (Fig. 3, top row, compare to Fig. 1). However, addition of apyrase to both the apical and basolateral baths completely abolished wave propagation as defined by the absent Ca^2+ responses at ROI 70 and 140, without affecting carbachol-induced responses (Fig. 3, lower row). The Ca^2+ responses in the stimulated cell were markedly (~70%) reduced, but not completely prevented with bilateral apyrase. Thus, we conclude that apyrase-sensitive nucleotides released from polarized epithelial cells into both extracellular compartments participated in the Ca^2+ wave propagation.
Evidence for Nucleotide (P2) Receptors in Mediating Ca\textsuperscript{2+} Waves

We next investigated whether the released nucleotides interacted with P2-receptors to generate intercellular Ca\textsuperscript{2+} waves. To test for expression of P2-receptors in mouse airway epithelia, we characterized nucleotide-induced Ca\textsuperscript{2+} responses in our preparations (Fig. 4 a). ATP and UTP were effective on both apical and basolateral surfaces, whereas ADP was more effective on the basolateral surface. The equipotency of ATP and UTP suggested P2Y\textsubscript{2}-receptor expression on the apical membrane (Nicholas et al., 1996). The effectiveness of ADP in addition to ATP/UTP at the basolateral surface suggested expression of both P2Y\textsubscript{1} and P2Y\textsubscript{2}-receptors at this barrier (see below).

To determine whether P2-receptor activation was required for generation of intercellular Ca\textsuperscript{2+} waves, homologous desensitization protocols were performed. Selective pretreatment of the apical surface with ATP (300 μM) attenuated, but did not prevent, the propagation of Ca\textsuperscript{2+} signals as compared with control: Δ[Ca\textsuperscript{2+}] in ROI\textsubscript{70} 12 s after mechanical stimulation after apical ATP pretreatment was 35 ± 20 nM (n = 3) versus 171 ± 16 nM (n = 20, control). Similarly, selective pretreatment of the basolateral surface with ATP (300 μM) reduced, but did not abolish, Ca\textsuperscript{2+} wave propagation as compared with control: Δ[Ca\textsuperscript{2+}] in ROI\textsubscript{70} were 101 ± 10 nM, and 171 ± 16 nM; n = 3 and 20, respectively. In contrast, simultaneous exposure of P2-receptors on both epithelial surfaces to ATP abolished the propagation of Ca\textsuperscript{2+} waves (Δ[Ca\textsuperscript{2+}] in ROI\textsubscript{70} = 0.7 ± 2.8 nM; Fig. 4, b and c). As a test for the selectivity of ATP pretreatment, the carbachol-induced Ca\textsuperscript{2+} responses were tested at the end of each protocol and were not affected by this maneuver (Fig. 4 c).

To test whether the inhibitory effect of ATP pretreatment on Ca\textsuperscript{2+} waves occurred at the level of P2-receptor desensitization or downstream in the signaling pathway (e.g., due to desensitization of IP\textsubscript{3} receptors), the spread of mechanically induced Ca\textsuperscript{2+} waves was measured after carbachol pretreatment. No significant differences were found in mechanically induced Ca\textsuperscript{2+} waves after carbachol pretreatment as compared with control (Δ[Ca\textsuperscript{2+}] in ROI\textsubscript{70} were 140 ± 24 nM and 171 ± 16 nM, respectively; n = 5 and 20).

These results strongly suggest that extracellular nucleotides released into both apical and basolateral baths upon mechanical stimulation interact with P2-receptors to generate intercellular Ca\textsuperscript{2+} waves. Again, the Ca\textsuperscript{2+} response in the mechanically stimulated cell itself was substantially reduced by maneuvers that modulated extracellular nucleotide levels/P2-receptor sensitivity, indicating that the elevation in [Ca\textsuperscript{2+}], in that cell was dominated by nucleotide release, but also involved a second mechanism.

Adenine Nucleotides Released Across the Basolateral Barrier in Response to Mechanical Stimulation Interact with a P2-receptor

As a second test for the requirement for P2-receptors in Ca\textsuperscript{2+} wave propagation, and as a reagent to identify which released nucleotides participated in the Ca\textsuperscript{2+} wave propagation, we studied intercellular Ca\textsuperscript{2+} waves in nasal epithelial cells from P2Y\textsubscript{2}-R (−/−) mice (Homolya et al., 1999). Consistent with the absence of P2Y\textsubscript{2}-R expression, these cells exhibited no significant Ca\textsuperscript{2+} responses to nucleotides added to the apical bath, and the responses to UTP on the basolateral surface were abolished (Fig. 5 a). The equipotency and equiefficiency of ADP and ATP after basolateral administration suggested that the residual P2Y-receptor on the basolateral membrane of P2Y\textsubscript{2}-R (−/−) cells was the adenine-selective P2Y\textsubscript{1}-receptor. The observation that these responses were blocked by the specific P2Y\textsubscript{1}-
Mechanical stimulation of a single P2Y2-R (-/-) cell within a polarized culture induced Ca^{2+} waves (Fig. 5 b) that were significantly smaller in magnitude than in wild-type [P2Y2-R (+/+) ] cultures (Δ[Ca^{2+}] in ROI 70 were 106 ± 9 nM and 171 ± 16 nM, respectively; n = 11 and 20, P < 0.01). This reduction in magnitude likely reflected the contribution of P2Y2-R to this response.

In contrast to wild-type cells, the propagation of Ca^{2+} signals was abolished in P2Y2-R (-/-) cells by selective basolateral pretreatment with ATP or by basolateral administration of PAPS (Fig. 5 c). The carbachol-induced Ca^{2+} responses again were not significantly different after ATP pretreatment (201 ± 48 nM) or in the presence of PAPS (127 ± 8 nM) from those in control experiments (187 ± 47 nM), indicating that the inhibitory effect of ATP or PAPS was specific to basolateral P2Y1-receptors. These data are consistent with the notion that apical mechanical stimulation of P2Y2-R (-/-) cells induced Ca^{2+} waves by the basolateral release of adenine nucleotides interacting with basolateral P2Y1-receptors.

Figure 4. Participation of P2-receptors in intercellular Ca^{2+} waves. a, Functional expression of P2Y-receptors in polarized mouse epithelial cells. Nucleotide-induced Ca^{2+} responses to apical versus basolateral additions of ATP (squares), UTP (circles), ADP (upright triangles), and UDP (inverted triangles) were measured. Basal to peak changes in [Ca^{2+}], levels (mean ± SEM) are plotted (n = 3–6/point). b, Inhibition of intercellular Ca^{2+} wave by desensitization of P2-receptors. Bilateral pretreatment with ATP (300 μM) completely prevented the propagation of Ca^{2+} signals without affecting the carbachol-induced Ca^{2+} response. c, Quantitative analysis of the inhibition of intercellular Ca^{2+} waves: Time course of [Ca^{2+}], for the three regions of interest in the same experiment shown (left) and mean change in Ca^{2+} levels (± SEM) for several similar experiments (n = 15) (right). For details see legend of Fig. 1 c.

Uridine Nucleotides Are also Released from Murine Airway Epithelia

Next, we studied whether UTP also contributed to the propagation of mechanically induced Ca^{2+} waves. Our approach was to test whether the expression of a UTP-specific receptor in an airway epithelial system that did not exhibit Ca^{2+} waves would reconstitute this response. P2Y2-R (-/-) cells deficient in native UTP-sensitive purinoceptors in the presence of basolateral PAPS to block P2Y1-receptors do not exhibit Ca^{2+} waves in response to mechanical stimulation (see Fig 5 c, right). We infected this cell line with a retrovirus containing the cDNA of the human P2Y4-receptor, which is highly selective for UTP.
over ATP or UDP (Communi et al., 1995; Nguyen et al., 1995; Lazarowski et al., 1997b). The functional expression and characterization of the P2Y4-receptor was verified by the nucleotide-induced Ca\(^{2+}\) responses (Fig. 6 a). The cells transduced with the Hygro\(^{+}\)-only vector (Control) showed no significant Ca\(^{2+}\) responses to nucleotides, but exhibited carbachol responses (left). In contrast, UTP, but not other triphosphate nucleotides nor UDP, stimulated substantial elevations in cytosolic Ca\(^{2+}\) level in P2Y\(_{4}\)-transduced cells (right). The UTP-induced Ca\(^{2+}\) response was not affected by the P2Y\(_{1}\)-receptor antagonist (PAPS): \(\Delta\left[\text{Ca}^{2+}\right]_i\) were 130 ± 38 nM and 124 ± 28 nM in response to 1 \(\mu\)M UTP in the presence and absence of 100 \(\mu\)M PAPS (\(n = 3\) and 4), respectively.

Mechanical stimulation of a single cell did not induce Ca\(^{2+}\) wave in the P2Y\(_{2}\) (−/−) cells transduced with the Hygro\(^{+}\)-only vector, when 100 \(\mu\)M PAPS was present in the basolateral bath (Fig. 6, b and c, left), whereas large Ca\(^{2+}\) waves were observed in P2Y\(_{4}\)-transduced cells (right). Desensitization of the P2Y\(_{4}\) receptors with bilateral UTP pretreatment abolished the Ca\(^{2+}\) wave propagation in the P2Y\(_{4}\)-receptor expressing cells: \(\Delta\left[\text{Ca}^{2+}\right]_i\) in ROI\(_{70}\) after UTP pretreatment was 1.27 ± 0.14 nM, \(n = 3\). These results strongly suggest that focal mechanical stimulation induced extracellular UTP release, which contributed to the generation of Ca\(^{2+}\) waves.

**Nucleotide-release and Ca\(^{2+}\) Waves in Human Airway Epithelia**

To assess the relevance of our findings in mice to other species, we studied human bronchial epithelial cells in well-differentiated, polarized cultures. These cells also exhibited mechanically induced intercellular Ca\(^{2+}\) waves (Fig. 7 a). Wave spread was attenuated by apical addition of apyrase and completely abolished by bilateral treatment with apyrase (Fig. 7 b), indicating that the phenomenon of bilateral 5\('\) nucleotide-release observed in the mouse model systems was relevant to human airway cells.

**Discussion**

Unlike previous studies (Boitano et al., 1992; Hansen et
al., 1993; Sneyd et al., 1995; Sanderson, 1996; Dirksen 1998; Felix et al., 1998), we grew airway epithelial cells as polarized monolayers on permeable membrane supports. This culture strategy yields an airway epithelial morphology that closely mimics in vivo morphology and experimentally permits access for drugs/reagents to both epithelial surfaces (Matsui et al., 1998a).

We demonstrated initially that for nonpolarized airway epithelial cells grown in discontinuous culture, i.e., islands (Matsui et al., 1997), cell–cell contact was not required for mechanically induced intercellular Ca$^{2+}$ waves in airway epithelial cells, consistent with a role for a diffusible substance as had been reported by others (Fig. 2). We extended this concept to studies that asked whether Ca$^{2+}$ waves were transmitted across physical gaps created in polarized, confluent airway epithelia. Ca$^{2+}$ waves jumped across these gaps, indicating a role again for a diffusible substance when airway epithelia are polarized.

A series of experiments (Figs. 2, 3, and 7) revealed that the diffusible substance(s) were sensitive to apyrase, an enzyme that cleaves 5'-purine and pyrimidine nucleotides into monophosphate nucleotides that are not recognized by P2Y-receptors. These data thus indicate a role for 5'-triphosphate nucleotides in this response. Further investi-
Bilateral Nucleotide-release from Epithelial Cells

Homolya et al.

Mechanical stimulation revealed that both adenine and uridine 5' nucleotides were released in response to mechanical stimulation and that the release occurred both ipsilateral and contralateral to the mechanical stimulus. The contribution of adenine nucleotides to Ca\(^{2+}\) wave propagation was demonstrated by the experiments with the P2Y\(_2\)-R (-/-) cells, in which a residual adenine nucleotide-selective receptor (P2Y\(_1\)) expressed on the basolateral membrane was activated by adenine nucleotides released across that barrier (Fig. 5). The participation of uridine nucleotides (UTP) in this response was demonstrated in studies with P2Y\(_2\)-R (-/-) cells reconstituted with the UTP-specific hP2Y\(_4\)-R (Fig. 6). The observation that apyrase was required in both baths to block mechanically induced Ca\(^{2+}\) waves in wild-type cells (Fig. 3) and P2Y\(_2\)-R (-/-) cells expressing hP2Y\(_4\) (Fig. 6) demonstrated that both adenine and uridine nucleotides were released across both barriers.

The involvement of P2-receptors in sensing released nucleotides and triggering Ca\(^{2+}\) release was likewise demonstrated in a series of studies. Pretreatment with ATP to induce homologous desensitization abolished Ca\(^{2+}\) waves, consistent with a role for P2Y-receptors (Fig. 4). The contribution of the P2Y\(_2\)-receptor to the epithelial response was studied using P2Y\(_2\)-R deficient nasal cells and revealed a 40–50% reduction in responses in cells without P2Y\(_2\)-R as compared with wild-type cells (Figs. 5 c, left and 1 c, right). The contribution of the P2Y\(_1\)-receptor on the basolateral membrane of murine airway epithelia was demonstrated by the virtual abolition of Ca\(^{2+}\) waves in P2Y\(_2\)-R deficient cells when the basolateral P2Y\(_1\)-R was blocked with the P2Y1-R antagonist PAPS (Fig. 5 c, right) or desensitized by ATP pretreatment. Since only bilateral maneuvers resulted in complete inhibition of mechanically induced Ca\(^{2+}\) waves in wild-type cells (Figs. 3, 4, b–c, and 7 b), we conclude that P2-receptors on both epithelial surfaces contribute to Ca\(^{2+}\) wave formation.

Despite previous reports that blockers of gap junctional communication inhibited intercellular Ca\(^{2+}\) waves in airway epithelia (Sanderson et al., 1990; Boitano et al., 1992, 1998) and other cell types (Tordjmann et al., 1997; Toyofuku et al., 1998), our data provide little evidence for a role for IP\(_3\) permeation through gap junctions in mediating Ca\(^{2+}\) waves in polarized airway epithelia. Further, our polarized epithelial preparations exhibited little gap junctional communication as evidenced by Lucifer yellow and calcein dye transfer measurements (data not shown), which mimics the paucity of gap junctions reported in mature airway epithelia in vivo (Carson et al., 1989). Further, a requirement for IP\(_3\) permeation through gap junctions in Ca\(^{2+}\) wave propagation was ruled out on the basis of our experiments with discontinuous cultures that demonstrated wave transmission was mediated by a diffusible,
apyrase-sensitive substance (Fig. 2). The previous failures to block Ca\textsuperscript{2+} waves by maneuvers that were designed to reduce nucleotide concentrations and/or P2Y-receptor activation (Hansen et al., 1993) in confluent airway epithelial cultures grown on glass coverslips likely reflects the failure to gain access to the basolateral surface of cells to interdict the extracellular nucleotide-release/actions at that barrier. A recent publication indicated that several connexin proteins can influence ATP-release from some cells (Cotrina et al., 1998). These experiments may point to a role for gap junctional channels in release of a soluble mediator (nucleotides) rather than cell–cell permeation of a diffusible messenger (IP\(_3\)).

We also noted that the mechanically stimulated cell exhibited a rise in Ca\textsuperscript{2+}, likely in response to two mechanisms. First, all maneuvers that either cleaved extracellular nucleotides and/or blocked or deleted P2-receptors reduced the response in the stimulated cell by ~60% (Figs. 2–7). Therefore, there was an autocrine effect of nucleotides on the stimulated cell. With respect to a nucleotide-independent mechanism mediating this response, others have suggested that it may reflect direct activation of plasma membrane Ca\textsuperscript{2+} influx pathways, perhaps via stretch-activated cation channels (Hansen et al., 1993; Boitano et al., 1994; Felix et al., 1996).

The mechanism of nucleotide-release in airway epithelial cells in response to mechanical stimuli is not understood. There is a controversy over whether the cystic fibrosis transmembrane conductance regulator (CFTR) acts in part as an ATP-channel and/or regulates an ATP-transporter in airway epithelia (Reisin et al., 1994; Schiewbert et al., 1995; Reddy et al., 1996; Abraham et al., 1997; Devidas and Guggino, 1997; Grygorczyk and Hanrahan, 1997; Watt et al., 1998). Since CFTR is expressed selectively in the apical membrane of airway epithelial cells (Schiewbert et al., 1995), our observation that nucleotide-release is bilateral makes it unlikely that CFTR contributes as an ATP-release pathway to the mechanically induced intercellular Ca\textsuperscript{2+} waves in polarized airway epithelia. Further, no role for CFTR in ATP-release has been proposed.

The model shown in Fig. 8 summarizes these observations. Apical mechanical stimulation of a cell in a polarized epithelium resulted in the ipsilateral and contralateral release of 5'-triphosphate nucleotides (ATP and UTP) that activated in an autocrine manner the stimulated cell and in a paracrine fashion adjacent cells via P2Y-receptors (P2Y\(_1\) and P2Y\(_2\)). Our studies leave the question open whether nucleotide-diphosphates also contribute to Ca\textsuperscript{2+} wave propagation, it being possible that nucleotide-diphosphates are directly released from cells or appear as the hydrolytic products of released triphosphates.

Wave propagation could reflect nucleotide release from mechanically stimulated cells as the sole source (point source), or be mediated in part by a regenerating (nucleotide-induced nucleotide release) mechanism. As a test of the regenerating signal hypothesis, we used luciferin/luciferase to assay for UTP-induced ATP-release from wild-type mouse nasal cells and could detect no release (data not shown). The observation that the kinetics of Ca\textsuperscript{2+} wave propagation in a discontinuous culture were not different on the stimulated and opposite sides of the gap (Fig. 2b) is more consistent with release from a point source. The model (Fig. 8) accordingly describes the nucleotide source as exclusively from the mechanically stimulated cell (point source) as the simplest hypothesis. As part of our analysis of this hypothesis, we estimated the quantity of ATP required to be released from a point source to mediate the Ca\textsuperscript{2+} wave responses shown in Fig. 1. We assumed two hemispheric distributions of nucleotides in the bulk solution above the apical and beneath the basolateral cultures surfaces, and compared the measured ΔCa\textsuperscript{2+} responses at ROIs 70 and 140 (Figs. 1, 3, and 5c) to the corresponding dose–effect relationships for nucleotide-induced ΔCa\textsuperscript{2+} (Figs. 4a and 5a) to estimate the profile of nucleotide concentrations at the culture surface (Larsen et al., 1992). These calculations indicated that ~6–9 fmol ATP (~20% of the total cellular ATP content of a single cell) would be required to produce the magnitude or pattern of responses observed.

In conclusion, these studies demonstrated that a response of airway epithelia to local mechanical stress is the production of intercellular Ca\textsuperscript{2+} waves mediated by release of nucleotides into the extracellular space that interact with P2Y-receptors. The Ca\textsuperscript{2+} wave propagation in polarized airway epithelia does not require intercellular IP\(_3\) permeation. Thus, polarized airway epithelia behave, in general, similarly to other cell types by producing intercellular signaling through extracellular nucleotide-release.

From the organ-level physiologic perspective, the generation of Ca\textsuperscript{2+} waves within airway epithelia coordinates intracellular defense mechanisms on airway surfaces (e.g., salt and water transport, ciliary beat frequency, and mucin secretion; Mason et al., 1991; Lethem et al., 1993; Geary et al., 1995) in response to mechanical stress. Our studies extend the spectrum of extracellular nucleotide signaling (Burnstock, 1997; Ferguson et al., 1997) by demonstrating that local mechanical stress induces release of both adenine and uridine nucleotides, which suggests that cells expressing uridine receptors (e.g., P2Y\(_2\), P2Y\(_4\), and/or P2Y\(_6\)-R) can respond to the epithelial-derived signals. Importantly, we demonstrate that epithelia can transduce the information of luminal (apical) mechanical stress into a signal (5'-nucleotides) released into the contralateral compartment. We speculate that basolateral release of nucleotides may be useful not only for coordination of local interepithelial cell responses to mechanical stresses, but also serves as a paracrine signal coordinating the responses of cells (e.g., nerves and inflammatory cells) in the submucosal space of the airway wall.

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