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 basolateral 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 airway 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). Fur-

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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 intercellular Ca^{2+} waves in airway epithelia. We generated unique airway epithelial model systems, including nasal epithelial cell lines from wild-type and $P2Y_2$-R $(-/-)$[1] ($P2Y_2$-receptor deficient mice) and $P2Y_2$-R $(-/-)$ cells reconstituted with a P2Y-receptor specific for UTP (human $P2Y_4$; Communi et al., 1995; Nguyen et al., 1995; Lazarowski et al., 1997b), to investigate this hypothesis. Importantly, most cultures were grown on polarized supports that allowed access to apical or basolateral surfaces for selective addition of reagents.

Materials and Methods

Airway Epithelial Cell Cultures

Immortalized nasal epithelial cells obtained from normal and $P2Y_2$-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 5×10^5 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 (R_t) with an EVOM (WPI). Mean R_ts were $344 \pm 31\ \Omega\ cm^2$, $n = 122$.

For studies with nonpolarized cultures, the cells were seeded on glass coverslips previously coated with 0.3 mg/ml Vitrogen, and cultured for 6–8 h.

To generate a $P2Y_2$-R $(-/-)$ cell line expressing the human $P2Y_4$-receptor, the plasmid harboring the DNA sequence of the human $P2Y_4$-receptor was obtained from Drs. T.K. Harden and R.A. Nicholas (The University of North Carolina, Chapel Hill, NC). A retroviral expression vector was generated as described previously (Comstock et al., 1997). For infection of the $P2Y_2$-R $(-/-)$ mouse epithelial cell line, the cells were seeded at 3×10^4 cells/cm^2 density, cultured overnight, incubated with the retrovirus and polybrene (8 μg/ml) for 2 h, and were then washed. For selection, the culture was maintained in medium containing 100 μg/ml hygromycin for 14 d, a time/concentration sufficient to kill all cells in mock-infected cultures. For control experiments, a cell line was generated by transducing the $P2Y_2$-R $(-/-)$ mouse epithelial cell line with a retroviral vector expressing *Hygro*^{r} alone (Comstock et al., 1997).

For primary human airway cultures, bronchial epithelial cells from lung transplant donors were processed according to protocols as described elsewhere (Matsui et al., 1998a). For imaging studies, disaggregated airway epithelial cells were seeded at 10^6 cells/cm^2 density on Transwell Col filters in Ham's F12-based medium supplemented with 5 ng/ml insulin, 500 ng/ml hydrocortisone, 10 ng/ml epidermal growth factor, 3.75 μg/ml endothelial cell growth substance, and 1 mM Ca^{2+} (total). The human cultures were studied 10–12 d after seeding when significant R_ts were established ($x = 66 \pm 20\ \Omega cm^2$, $n = 11$).

$Ca^{2+}{}_i$ Measurements

The cell cultures were washed with hormone free Ham's F12 medium and incubated with 5 μM Fura-2 AM for 30 min at 37°C in the presence of verapamil (40 μM), then were washed twice with Ringer solution (130 mM Na^+, 128 mM Cl^-, 5 mM K^+, 1.3 mM Ca^{2+}, 1.3 mM Mg^{2+}, 5 mM glucose, and 10 mM Hepes; pH 7.4). Digital fluorescence ratio imaging was performed with a RatioMaster-D video imaging system from Photon Technology, Inc. A Zeiss Axiovert 35 microscope and a Nikon UV-F 100× (1.3) glycerol immersion objective were used. The cells were alternately illuminated with 340- and 380-nm excitation light (band widths 6 nm), and a pair of fluorescence images (average of 8 video frames) were acquired every 1.8 seconds (>450 nm).

A micropipette affixed to a micromanipulator was used to deliver mechanical stimulation to a single cell within the epithelial sheet. The pipette tip (0.8–1-mm diameter) was positioned above the apical membrane of the cells, and slowly advanced in ~3–5-μm increments until a Ca^{2+} signal was observed. The micropipette was immediately withdrawn after stimulation. Background images were obtained after each experiment by a quenching technique that used digitonin (40 μM) and $MnCl_2$ (4 mM). The optical parameters of the system, the R_{max}, R_{min}, and K_d 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 apyrase 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 ($[Ca^{2+}]_i$) 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. 1 a). The viability of each cell after mechanical stimulation was demonstrated by impermeability to ethidium-homodimer (Fig. 1 b), the persistence of receptor-mediated Ca^{2+} responses elicited by the muscarinic recep-

[1]*Abbreviations used in this paper:* $[Ca^{2+}]_i$, intracellular calcium concentration; CFTR, cystic fibrosis transmembrane conductance regulator; $P2Y_2$-R $(-/-)$, $P2Y_2$-receptor deficient mice; PAPS, adenosine 3′-phosphate 5′-phosphosulfate; ROI, region of interest.

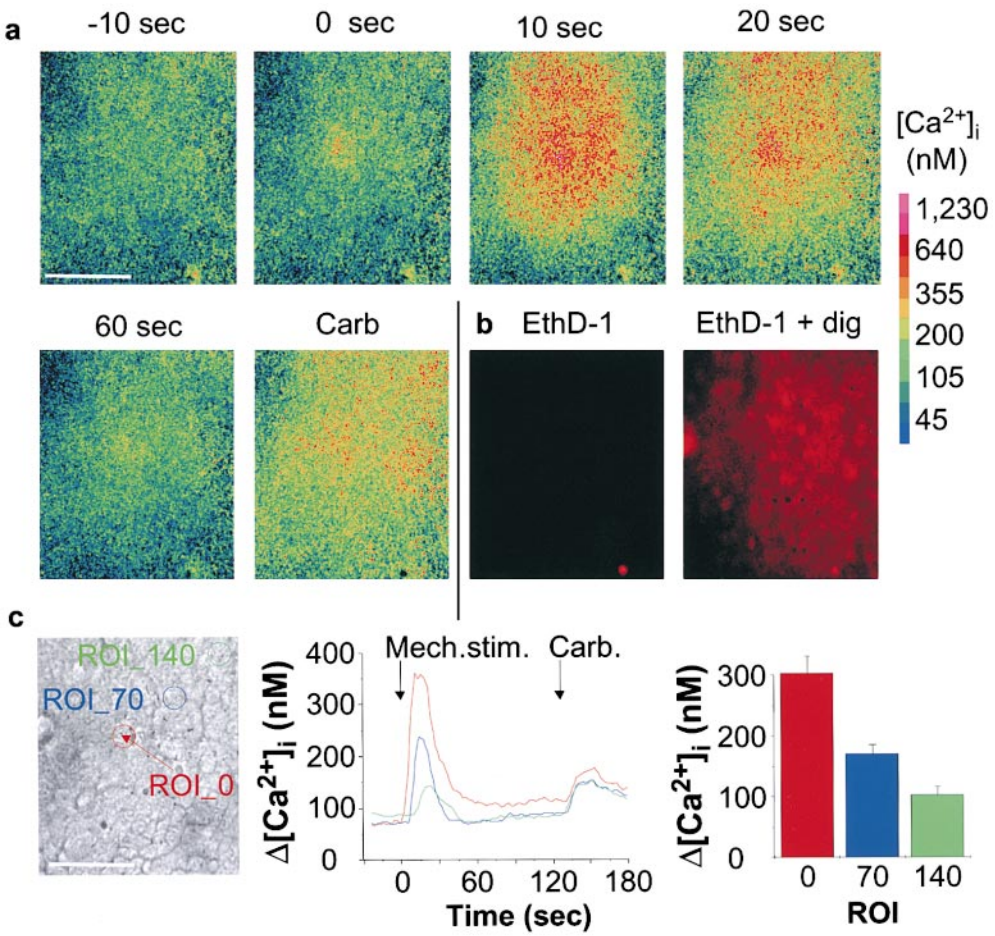

Figure 1. Propagation of Ca^{2+} signals in polarized cultures of normal mouse nasal epithelial cells. a, Ca^{2+} waves: a single cell in a confluent, Fura-2 loaded culture grown on a permeable membrane support was mechanically stimulated with a micropipette. This stimulation resulted in a radially propagating Ca^{2+} wave. The numbers above the pseudocolor images indicate the time in seconds relative to the stimulus. The first image in which an elevation in Ca^{2+} level was observed is considered as time 0. Subsequent exposure to 1 mM carbachol (Carb) induced a second Ca^{2+} response in the cells (including the stimulated one), showing that the cells remain viable and responsive. Color bar shows the calibration for $[Ca^{2+}]_i$. Bar, 100 μm. b, Cell viability: viability of the cells after mechanical stimulation was also tested by the addition of 10 mM ethidium-homodimer (EthD-1). Only one cell (distinct from the mechanically stimulated one) showed dye uptake after a 5-min incubation. As control for the viability assay, all the cells were lysed with 80 M digitonin in the continuous presence of the dye (EthD-1 + dig). c, Quantitation of Ca^{2+} waves: Ca^{2+} concentrations were quantitated in three circular regions of interest 70 μm apart (ROI_0, ROI_70 and ROI_140) shown in the bright-field image (left). An arrow indicates the stimulated cell. Bar, 100 μm. Time course of $[Ca^{2+}]_i$ for the three regions of interest in the same experiment is shown in the central panel. The color of each tracing corresponds to the color of circles denoting each ROI shown in the bright-field image on the left. The right panel depicts the mean change in Ca^{2+} levels (± SEM) for the ROI_0, ROI_70 and ROI_140 at 8, 12, and 16 s after the stimulus, respectively ($n = 20$).

tor agonist carbachol (see Fig. 1, a and c), and the absence of Fura-2 leakage.

To quantitate wave propagation, $[Ca^{2+}]_i$ 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 Ca^{2+} response was greatest in the stimulated cell and declined as a function of time and distance from this cell (middle). The 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 $[Ca^{2+}]_i$ at times of peak responses were used for calculating and plotting mean maximum Ca^{2+} responses (right).

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

We used two approaches to test whether the intercellular 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 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 Ca^{2+} signal to the adjacent cells (bottom row), suggesting that the mediator of the Ca^{2+} wave was a 5′ nucleotide. The carbachol-induced 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 Ca^{2+} wave skipped over the gap (Fig. 2 b, top row), indicating involvement of a released,

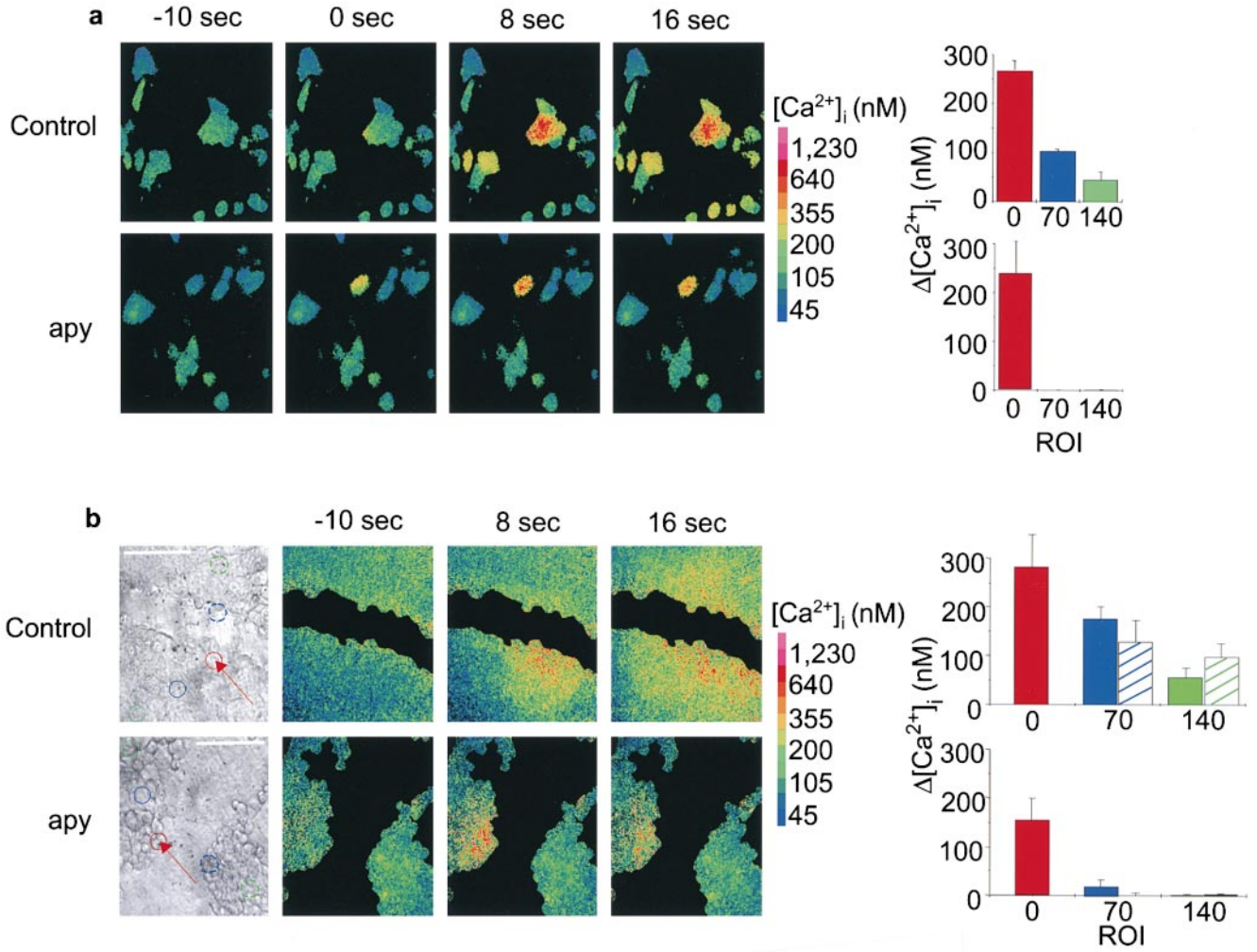

Figure 2. Cell–cell contact is not required for the mechanically induced Ca^{2+} wave in mouse nasal epithelial cells. a, Propagating Ca^{2+} waves in not-contacting cells. Mechanical stimulation of a single cell in a nonpolarized culture grown on a glass coverslip resulted in a Ca^{2+} wave propagation (top). Elevation in Ca^{2+} level was observed in cells that were not in physical contact with the stimulated cell. Apyrase (10 U/ml) abolished the spread of Ca^{2+} wave (bottom). Low intensity pixels (<32) of the background were omitted on the pseudocolor images. Bar graph on the right depicts summary data for multiple experiments (n = 4 and 6). For other details, see Fig. 1 c. b, The Ca^{2+} wave propagates over a physical gap: a confluent culture of mouse epithelial cells grown on permeable membrane support was gently scraped with a micropipette 1 h before the experiment to produce a gap in the epithelial sheet. A single cell close to the scrape (marked with a red arrow on the bright-field image on the left) was mechanically stimulated in the absence (top) and presence (bottom) of apyrase (10 U/ml, grade V). The Ca^{2+} wave propagated over the gap (top) indicating that the signal transmission mechanism involves extracellular substance(s). The mediator was sensitive to apyrase (bottom). Bar, 100 μm. To avoid artificial background noise effects of low intensity fields, the gap was masked on the basis of the bright-field image. The mean changes in Ca^{2+} level (± SEM) are shown in the bar graph in the right panels (n = 4 and 4, respectively). The solid bars refer to the ROIs on the stimulated side, whereas hatched bars refer to the ROIs on the opposite side of the gap. No significant differences between the two sides were found in the spread of the Ca^{2+} wave.

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. 2 b, 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.

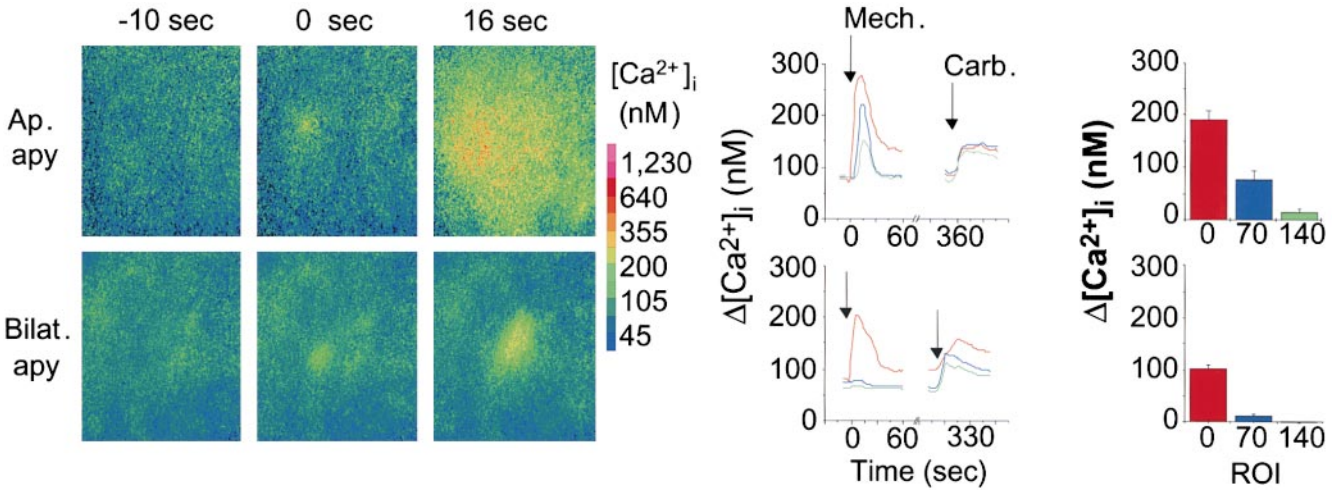

Figure 3. Inhibition of intercellular Ca^{2+} waves with extracellular nucleotidase activity. Pseudocolor images on the left demonstrate the intracellular Ca^{2+} levels at designated time points in representative experiments. In the center, time courses of $[Ca^{2+}]_i$ in these experiments are shown. Summary data for multiple experiments are shown on the right (n = 6 and 13). Apical addition of apyrase (10 U/ml) attenuated, but did not block, the mechanically induced intercellular Ca^{2+} wave (top), whereas bilateral addition of apyrase completely prevented the Ca^{2+} spread (bottom). Subsequent carbachol-induced Ca^{2+} responses were not affected as compared with control experiments shown in Fig. 1 c (middle).

Evidence for Nucleotide (P2) Receptors in Mediating Ca^{2+} Waves

We next investigated whether the released nucleotides interacted with P_2-receptors to generate intercellular Ca^{2+} waves. To test for expression of P_2-receptors in mouse airway epithelia, we characterized nucleotide-induced Ca^{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 $P2Y_2$-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 $P2Y_1$ and $P2Y_2$-receptors at this barrier (see below).

To determine whether P_2-receptor activation was required for generation of intercellular Ca^{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^{2+} signals as compared with control: $\Delta[Ca^{2+}]_i$ in ROI_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^{2+} wave propagation as compared with control: $\Delta[Ca^{2+}]_i$ in ROI_70 were 101 ± 10 nM, and 171 ± 16 nM; n = 3 and 20, respectively. In contrast, simultaneous exposure of P_2-receptors on both epithelial surfaces to ATP abolished the propagation of Ca^{2+} waves ($\Delta[Ca^{2+}]_i$ in ROI_70 = 0.7 ± 2.8 nM; Fig. 4, b and c). As a test for the selectivity of ATP pretreatment, the carbachol-induced Ca^{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^{2+} waves occurred at the level of P2-receptor desensitization or downstream in the signaling pathway (e.g., due to desensitization of IP_3 receptors), the spread of mechanically induced Ca^{2+} waves was measured after carbachol pretreatment. No significant differences were found in mechanically induced Ca^{2+} waves after carbachol pretreatment as compared with control ($\Delta[Ca^{2+}]_i$ in ROI_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 P_2-receptors to generate intercellular Ca^{2+} waves. Again, the Ca^{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 $[Ca^{2+}]_i$ 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 P_2-receptors in Ca^{2+} wave propagation, and as a reagent to identify which released nucleotides participated in the Ca^{2+} wave propagation, we studied intercellular Ca^{2+} waves in nasal epithelial cells from $P2Y_2$-R (−/−) mice (Homolya et al., 1999). Consistent with the absence of $P2Y_2$-R expression, these cells exhibited no significant Ca^{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 $P2Y_2$-R (−/−) cells was the adenine-selective $P2Y_1$-receptor. The observation that these responses were blocked by the specific $P2Y_1$-

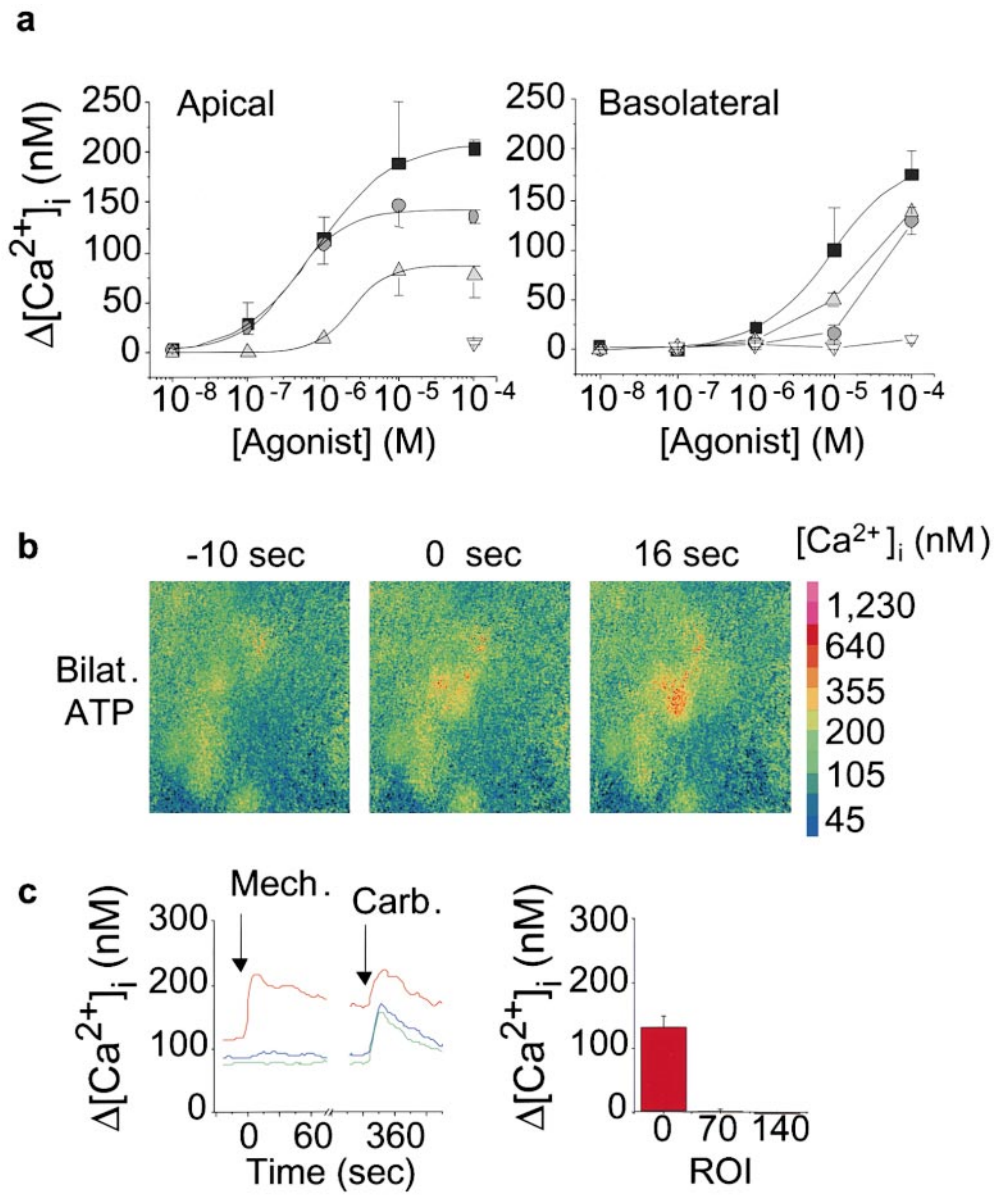

Figure 4. Participation of P_2-receptors in intercellular Ca^{2+} waves. a, Functional expression of P2Y-receptors in polarized mouse epithelial cells. Nucleotide-induced Ca^{2+} responses to apical versus basolateral additions of ATP (squares), UTP (circles), ADP (upright triangles), and UDP (inverted triangles) were measured. Basal to peak changes in $[Ca^{2+}]_i$ levels (mean ± SEM) are plotted (n = 3–6/point). b, Inhibition of intercellular Ca^{2+} wave by desensitization of P_2-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 $[Ca^{2+}]_i$ 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.

receptor antagonist adenosine 3′-phosphate 5′-phosphosulfate (PAPS; 100 μM; Boyer et al., 1996) was consistent with this identification.

Mechanical stimulation of a single $P2Y_2$-R (−/−) cell within a polarized culture induced Ca^{2+} waves (Fig. 5 b) that were significantly smaller in magnitude than in wild-type [$P2Y_2$-R (+/+)] cultures ($\Delta[Ca^{2+}]_i$ 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 $P2Y_2$-R to this response.

In contrast to wild-type cells, the propagation of Ca^{2+} signals was abolished in $P2Y_2$-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 $P2Y_1$-receptors. These data are consistent with the notion that apical mechanical stimulation of $P2Y_2$-R (−/−) cells induced Ca^{2+} waves by the basolateral release of adenine nucleotides interacting with basolateral $P2Y_1$-receptors.

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. $P2Y_2$-R (−/−) cells deficient in native UTP-sensitive purinoceptors in the presence of basolateral PAPS to block $P2Y_1$-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 $P2Y_4$-receptor, which is highly selective for UTP

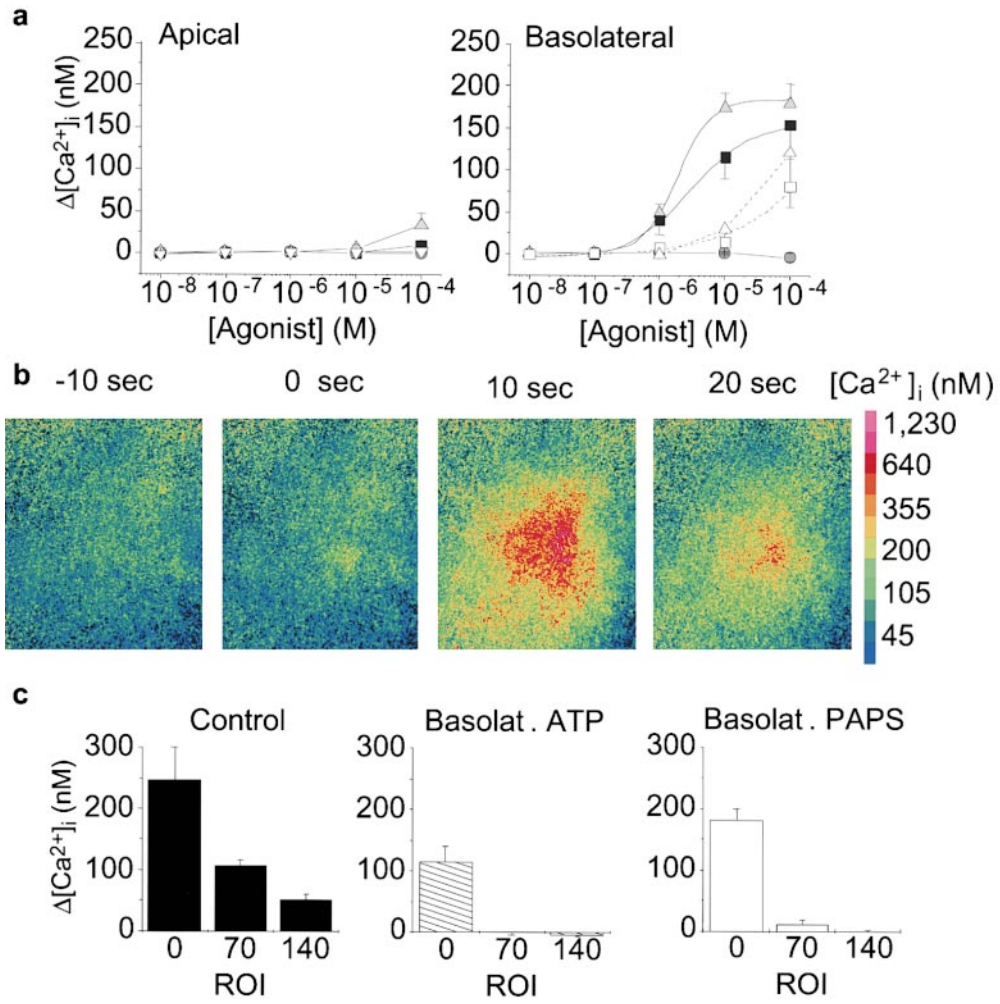

Figure 5. Propagating Ca^{2+} waves in $P2Y_2$-R (−/−) airway epithelial cells. a, Characterization of polarized cultures of nasal cells isolated from $P2Y_2$-receptor gene targeted (−/−) mice for residual expression of P2Y-receptors. Nucleotide-induced Ca^{2+} responses to ATP (squares), UTP (circles), ADP (upright triangles), and UDP (inverted triangles) were measured (n = 3–6/point). The effects of ATP (open squares) and ADP (open up triangles) were also assessed in the presence of 100 μM PAPS, a $P2Y_1$-receptor antagonist (Boyer et al., 1996). The pharmacological profiles indicate that the residual P_2-receptor (most likely $P2Y_1$-R) is expressed solely in the basolateral membrane. b, Mechanically induced intercellular Ca^{2+} wave in $P2Y_2$-R (−/−) cells in a representative experiment. The residual Ca^{2+} propagation in $P2Y_2$-R (−/−) cells suggests basolateral release of nucleotides interacting with $P2Y_1$-receptors in the basolateral membrane. For details see Fig 1 a. c, Inhibition of Ca^{2+} propagation in $P2Y_2$-R (−/−) cells. The magnitude of the Ca^{2+} waves was smaller in $P2Y_2$-R (−/−) cells as compared with wild-type cells (Fig. 1 c, right). Basolateral pretreatment with ATP (hatched bars) or PAPS (open bars) completely abolished the intercellular Ca^{2+} wave as compared with $P2Y_2$-R (−/−) cells without treatment (solid bars; n = 11, 6, and 9, respectively). For details for quantitative analysis see legend of Fig. 1 c.

over ATP or UDP (Communi et al., 1995; Nguyen et al., 1995; Lazarowski et al., 1997b). The functional expression and characterization of the $P2Y_4$-receptor was verified by the nucleotide-induced Ca^{2+} responses (Fig. 6 a). The cells transduced with the *Hygro*^{r}-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 $P2Y_4$-R transduced cells (right). The UTP-induced Ca^{2+} response was not affected by the $P2Y_1$-receptor antagonist (PAPS): $\Delta[Ca^{2+}]_i$ were 130 ± 38 nM and 124 ± 28 nM in response to 1 μM UTP in the presence and absence of 100 μM 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*^{r}-only vector, when 100 μM PAPS was present in the basolateral bath (Fig. 6, b and c, left), whereas large Ca^{2+} waves were observed in $P2Y_4$-R 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: $\Delta[Ca^{2+}]_i$ 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

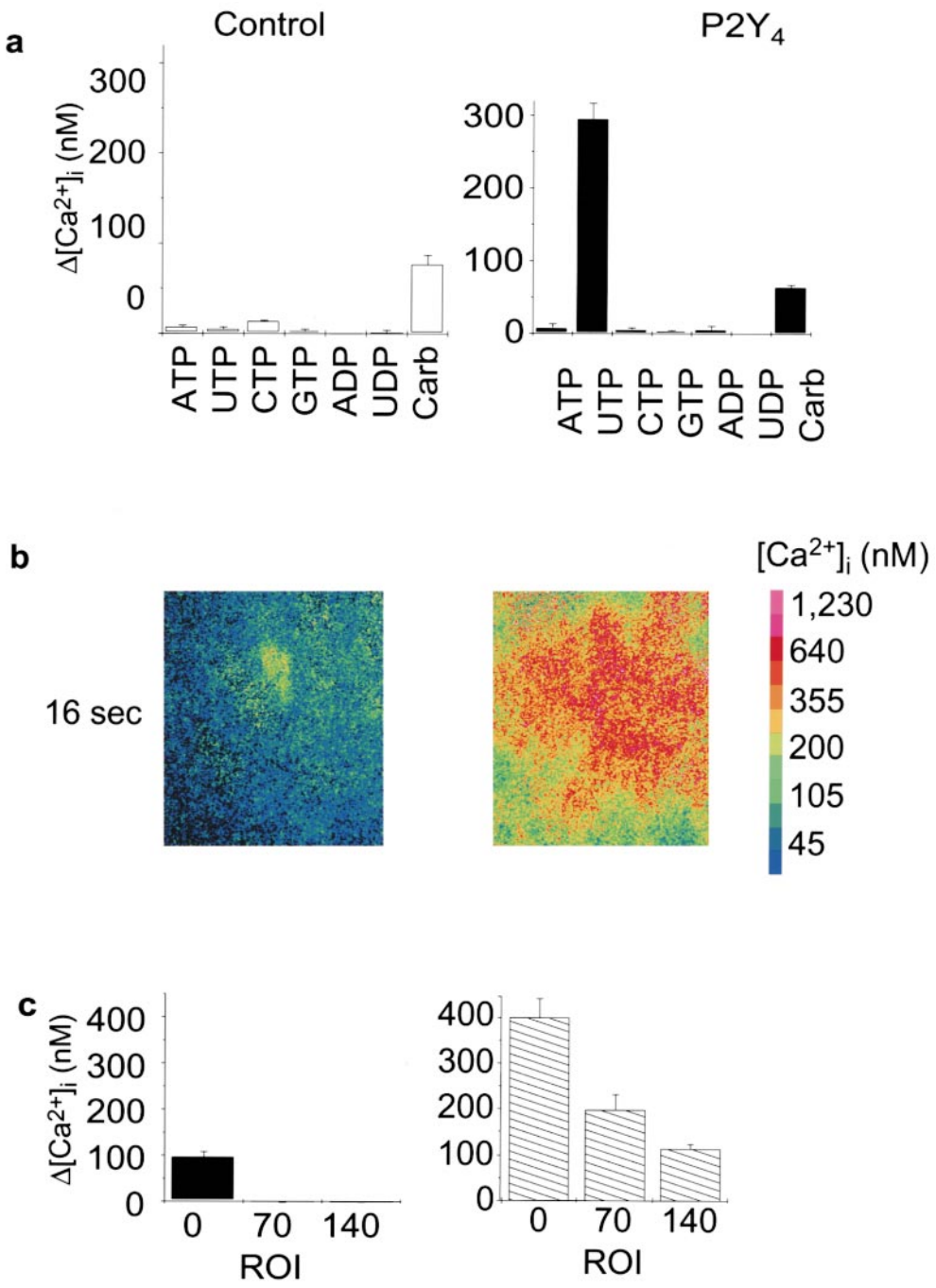

Figure 6. Participation of released UTP in the mechanically induced Ca^{2+} wave propagation. a, Characterization of a mouse $P2Y_2$-R (−/−) nasal cell line expressing the human $P2Y_4$-receptor. Ca^{2+} responses to nucleotides (100 μM) and carbachol (1 mM) were measured in $P2Y_2$-R (−/−) cells transduced with the *Hygro*^{r}-only vector (Control) and with the human $P2Y_4$-receptor ($P2Y_4$) (n = 3–4/point). Control [$P2Y_2$(−/−)] cells grown on glass coverslips showed no substantial Ca^{2+} responses to nucleotides but to carbachol. In contrast, UTP stimulated significant Ca^{2+} responses in $P2Y_4$-R transduced cells. No other nucleotide stimulated substantial Ca^{2+} response in the $P2Y_4$-receptor expressing cells. b, Mechanically induced intercellular Ca^{2+} wave in polarized culture of $P2Y_4$-R transduced cells in a representative experiment. Pseudocolor images demonstrate the intracellular Ca^{2+} levels in the empty vector-transduced $P2Y_2$(−/−) cells (left) and the $P2Y_4$-R transduced $P2Y_2$(−/−) cells (right) 16 s after local mechanical stimulation in the continuous presence of 100 μM PAPS in the basolateral bath. c, Quantitative analysis of Ca^{2+} wave propagation in control and $P2Y_4$-R transduced cells. Summary data for experiments similar to that shown in Fig 6 b. Mechanical stimulation of a single cell did not elicit Ca^{2+} wave in the $P2Y_2$(−/−) cells transduced with the *Hygro*^{r}-only vector, when 100 μM PAPS was present in the basolateral bath, whereas an extensive Ca^{2+} wave was observed in $P2Y_4$-R transduced cells (n = 3 and 3, respectively). For details for quantitative analysis see legend of Fig. 1 c.

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-

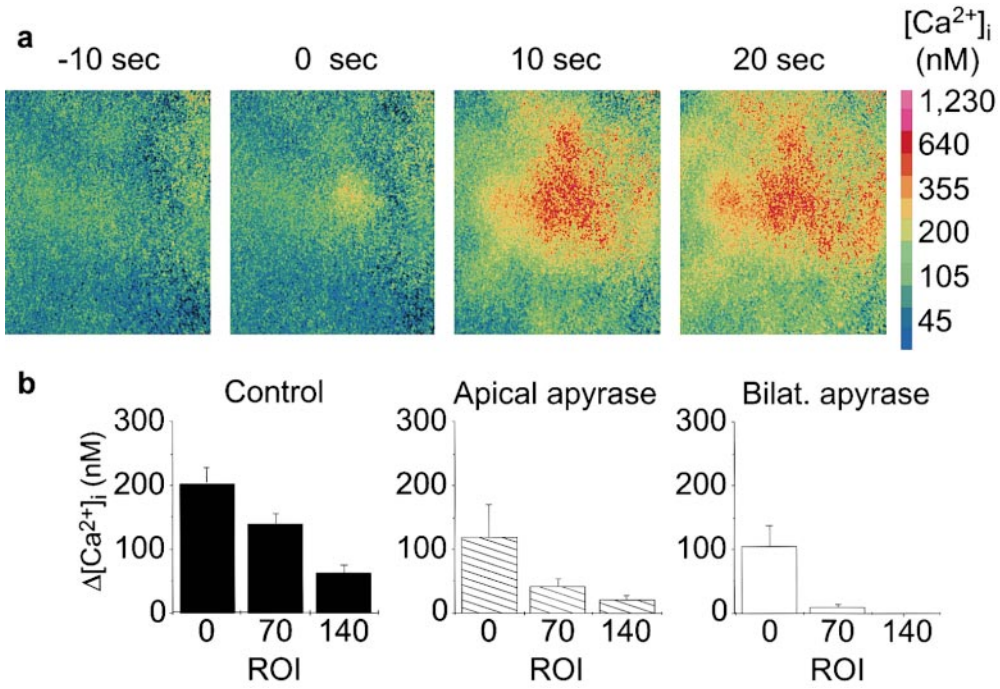

Figure 7. Intercellular Ca^{2+} waves in polarized human airway epithelial cells. a, A representative experiment. Local mechanical stimulation elicited cell to cell propagation of Ca^{2+} signals in well-differentiated human bronchial epithelial cell cultures. b, Block of Ca^{2+} wave propagation with extracellular nucleotidase activity. The intercellular Ca^{2+} waves were partially inhibited by apical addition of apyrase (hatched bars; $n = 5$) as compared with control (solid bars; $n = 3$). Bilateral treatment with apyrase completely abolished the spread of Ca^{2+} signals (open bars; $n = 10$).

gation 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.

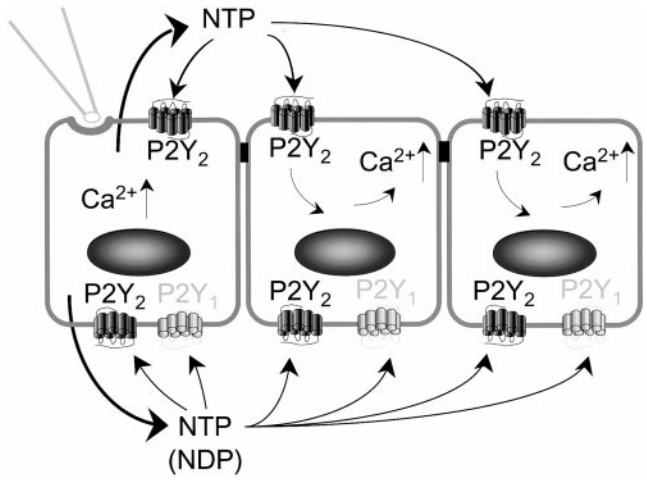

Figure 8. A proposed model for intercellular Ca^{2+} waves in airway epithelia. Mechanical stimulation on the apical surface of a polarized airway epithelial cell induces ipsilateral and contralateral release of nucleotides that activates the adjacent cells via $P2Y_2$-receptors on the apical membrane and via both $P2Y_1$- and $P2Y_2$-receptors on the basolateral membrane.

The involvement of P_2-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 $P2Y_1$-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 epithelial cells (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^{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^{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^{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; Schwiebert 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 (Schwiebert 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^{2+} waves in polarized airway epithelia. Further, no role for CFTR in UTP-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^{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^{2+} wave propagation in a discontinuous culture were not different on the stimulated and opposite sides of the gap (Fig. 2 b) 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^{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 $\Delta Ca^{2+}{}_i$ responses at ROIs 70 and 140 (Figs. 1, 3, and 5 c) to the corresponding dose–effect relationships for nucleotide-induced $\Delta Ca^{2+}{}_i$ (Figs. 4 a and 5 a) 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^{2+} waves mediated by release of nucleotides into the extracellular space that interact with P2Y-receptors. The Ca^{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^{2+} waves within airway epithelia coordinates intraepithelial 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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