Cell deformation at the air-liquid interface induces Ca\(^{2+}\)-dependent ATP release from lung epithelial cells

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Cell deformation at the air-liquid interface induces Ca\(^{2+}\)-dependent ATP release from lung epithelial cells. Am J Physiol Lung Cell Mol Physiol 300: L587–L595, 2011. First published January 14, 2011; doi:10.1152/ajplung.00345.2010.—Extracellular nucleotides regulate mucociliary clearance in the airways and surfactant secretion in alveoli. Their release is exquisitely mechanosensitive and may be induced by stretch as well as airflow shear stress acting on lung epithelia. We hypothesized that, in addition, tension forces at the air-liquid interface (ALI) may contribute to mechanosensitive ATP release in the lungs. Local depletion of airway surface liquid, mucins, and surfactants, which normally protect epithelial surfaces, facilitate such release and trigger compensatory mucin and fluid secretion processes. In this study, human bronchial epithelial 16HBE14o- and alveolar A549 cells were subjected to tension forces at the ALI by passing an air bubble over the cell monolayer in a flow-through chamber, or by air exposure while tilting the culture dish. Such stimulation induced significant ATP release not involving cell lysis, as verified by ethidium bromide staining. Confocal fluorescence microscopy disclosed reversible cell deformation in the monolayer part in contact with the ALI. Fura 2 fluorescence imaging revealed transient intracellular Ca\(^{2+}\) elevation evoked by the ALI, which did not entail nonspecific Ca\(^{2+}\) influx from the extracellular space. ATP release was reduced by ~40 to ~90% from cells loaded with the Ca\(^{2+}\) chelator BAPTA-AM and was completely abolished by N-ethylmaleimide (1 mM). These experiments demonstrate that in close proximity to the ALI, surface tension forces are transmitted directly on cells, causing their mechanical deformation and Ca\(^{2+}\)-dependent exocytotic ATP release. Such a signaling mechanism may contribute to the detection of local deficiency of airway surface liquid and surfactants on the lung surface.

mechanosensitive nucleotide release; airway liquid volume regulation; surfactant secretion

THE MUCOCILIARY CLEARANCE (MCC) system is the predominant innate defense mechanism of airways that removes noxious materials from the lungs. Its efficiency critically depends on the height and properties of a thin airway surface liquid (ASL) layer that covers the entire respiratory tract. It consists of two phases, the sticky mucus layer that entraps airborne particles and bacteria, inhibits bacterial growth, and protects airways from fluid loss, and the underlying low-viscosity phase of the periciliary liquid layer that provides a low-friction environment for ciliary beating. The rheological properties of both layers are tightly controlled by the coordinated secretion of ions, mucins, and water, which ultimately determine the local microenvironment on the mucosal surface. The requirement for proper mucus “conditioning” and ASL hydration on the epithelial surface is illustrated by pathological manifestations of cystic fibrosis lung disease characterized by viscous mucus that has lost its transportability, leading to mucostasis and chronic infections (7, 22, 23). Compelling evidence indicates that nucleotides in ASL, by interacting with purinergic receptors, provide endogenous signaling to MCC functions (14, 24), stimulate cilia beating, and coordinate fluid and mucin secretion (12, 18). Effective control of MCC by purinergic signaling necessitates nucleotide release onto the mucosal surface, but what is the trigger of such release and how it is linked to, e.g., deficient ASL volume or perturbations of its rheological properties, are poorly understood. Lung epithelial cells are subjected to many forms of mechanical stresses during the breathing cycle, including airflow shear stress and cell stretch. In vitro studies have shown that nucleotide release from airway epithelial cells is exquisitely sensitive to mechanical perturbations, such as cell culture plate tilting (9, 13), fluid shear stress (24), and hypotonic shock (2). Another form of mechanical stress, particularly relevant in the lungs, may involve cell distortion by tension forces at the air-liquid interface (ALI). Previous studies of T cells found that when a very thin film of liquid is drawn down on the cells, the ALI greatly deforms them (20). Epithelial cells are normally protected from such stresses by a gel-like layer of mucus in the airways, or a thin layer of surfactant-containing fluid in alveoli. However, in situations of low ASL volume, insufficient mucus, or surfactant content, especially when combined with shear stress, some cells may locally become “naked” and exposed to large tension forces at the ALI, which will cause their deformation. Mechanical cell deformation may trigger cellular ATP release, a potent surfactant, fluid, and mucin secretagogue.

In this in vitro study, we examined the hypothesis that reducing the volume of liquid covering lung epithelial cells will draw down a thin film of liquid on the cells to such proximity that tension forces at the ALI will cause cell deformation, intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) elevation, and Ca\(^{2+}\)-dependent ATP release. By briefly introducing an air bubble into the closed perfusion chamber or by culture dish tilting, cells were briefly exposed to ALI forces. We observed that such stimuli indeed produced reversible cell deformation (flattening), transient [Ca\(^{2+}\)]\(_{i}\) elevations, and concomitant Ca\(^{2+}\)-dependent ATP release that did not involve cell lysis. We propose that cell deformation in proximity to the ALI may provide a simple mechanism of sensing low ASL volume or low surfactant content on the lung mucosal surface, which will trigger mechanosensitive, Ca\(^{2+}\)-dependent ATP release and compensatory fluid secretion.
METHODS

Cells. Human lung carcinoma A549 cells were grown in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine. Human bronchial epithelial 16HBE14o- cells, a generous gift from Dr. D. Gruenert, were cultured as described elsewhere (3). All culture media constituents were from Gibco-BRL (Burlington, ON, Canada). Antibiotics (56 U/ml penicillin-G and 56 µg/ml streptomycin sulfate) were typically included in the culture media only in initial passages during cell culture expansion and were omitted in later passages of cells used in the experiments. ATP efflux was measured from cell monolayers grown to confluence (~500 A549 cells/mm²; ~600 16HBE14o- cells/mm²) on 24 x 60-mm glass coverslips or on cell culture-treated Costar six-well plastic plates (Corning, NY). Fura 2 calcium imaging experiments were performed with cells grown on circular 15-mm-diameter glass coverslips. To block exocytosis, the cells were pretreated with 1 mM N-ethylmaleimide (NEM) for 15 min at room temperature, and were examined at 37°C after washing.

Solutions and chemicals. Physiological isotonic solution (PS) contained (in mM): 140 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 glucose, and 10 TES, pH 7.4, adjusted with NaOH. For experiments in the absence of extracellular Ca²⁺, CaCl₂ was omitted and the solutions were supplemented with 0.1 mM EGTA to chelate trace Ca²⁺. All reagents, including Pluronic F127, NEM, and probenecid, were obtained from Sigma-Aldrich Canada (Oakville, ON, Canada). For calcium imaging, fura 2-AM was procured from Invitrogen-Molecular Probes (Kinston, ON, Canada).

ATP efflux assay. ATP efflux was measured with high temporal resolution in a custom-designed, low-volume (325 µl) flow-through chamber similar to that described previously (2) (Fig. 1A). With this low internal chamber volume and the perfusion rate of 1.3 ml/min, the time required to wash half of ATP content from the chamber (T½) was ~15 s. Briefly, a 24 x 60-mm coverslip with confluent cell monolayer was mounted in the chamber and perfused with a warm (37°C, inline SF-28 heater, Warner Instruments, Hamden, CT) solution (Fig. 1). The estimated shear stress generated by fluid flow of ~1.6 dyn/cm² was well below the threshold level of ≥8 dyn/cm² that induces detectable ATP release from lung epithelial cells (Grygorczyk R, Ptaszynski S, unpublished observations). The peristaltic pump may generate an extra pressure ΔP in the perfusion system and in the chamber, which may be transmitted onto the cells. However, ΔP was found to be near zero because the chamber had an open outlet, and its small fluctuations resulted mainly from hydrostatic pressure of a liquid present in the outlet tubing loop (~2 cmH₂O, see Fig. 1A). After equilibration in PS (15–30 min), to mechanically stimulate cells, perfusion was stopped and an air bubble was introduced through a separate entry port to completely fill chamber volume. After ~15 to 30 s, perfusion with PS was resumed and the air bubble was removed from the chamber. The 45° chamber inclination facilitated air bubble introduction and removal. The perfusate was collected continuously at 15- to 30-s intervals for later ATP content evaluations. During passage of an air bubble through the chamber, Fig. 1Aa, cells remain covered by only a thin fluid film, which exposes them to tension forces at the ALI. The net force acting perpendicularly to the cell surface will depend on cell apex curvature and will tend to flatten the cell (Fig. 1Ab, see discussion). In some experiments, cells were grown on six-well cell culture-treated plastic plates containing 2 ml PS/well and were stimulated by tilting the plate at 45° for 15 or 30 s under gentle warm air flow (~30–35°C) to mimic air flow in the airways (Fig. 1B). Tilting the plate results in gravity-driven draining flow, which will leave only a thin liquid layer covering cells on part of the cell monolayer and subject them to tension forces at the ALI. After 15 s in the horizontal position, the plate was tilted in the opposite direction to expose the second half of the cell monolayer. The plate was then returned to the horizontal position, media were gently mixed by orbital plate movement, and aliquots were collected for ATP content determinations. ATP in the samples was quantified by luciferase-luciferin assay, with ATP Assay Mix and ATP Assay Mix Dilution Buffer supplied by Sigma-Aldrich Canada. ATP release was then presented as concentrations (in nM) in media aliquots collected at different time points, or as cumulative release (in pmol/10⁶ cells) for 5 min after stimulation.

[Ca²⁺]ᵢ measurements. For [Ca²⁺]ᵢ measurements, cells were loaded (0.5–1 h, room temperature) with 10 µM fura 2-AM in physiological solution containing 0.02% Pluronic F127 and 2.5 mM probenecid, followed by 30-min deesterification in PS containing probenecid. For fluorescence imaging, a coverslip with fura 2-loaded cells was mounted in an imaging/perfusion chamber (RC-20, volume 48 µl) attached to a heated platform (P-5, Warner Instruments) on
stage of an inverted microscope (Nikon TE300). The imaging chamber was perfused continuously with a warm solution (37°C) via an inline heater (SF-28, Warner Instruments) at 0.5 ml/min to maintain shear stress below the threshold level for ATP release. The chamber had an additional port that served to introduce an air bubble to stimulate cells in a similar way as in ATP efflux assay. The cells were illuminated for 100 ms with alternate light wavelengths of 340 and 380 nm, with a high-pressure mercury lamp (100 W) via interference filters (Chroma Technology, Brattleboro, VT) mounted on a filter wheel (Sutter Lambda 10-C, Sutter Instrument, Novato, CA) and a dichroic mirror (510/540 nm, Chroma Technology). Fluorescence images were recorded at 15-s intervals with a digital camera and stored for later analysis. Fura 2 measurements are presented as the fluorescence F340/F380 ratio. In some experiments, to chelate [Ca2+], cells were loaded with BAPTA-AM (25 μM) for 30 min at room temperature in physiological solution.

Cell viability test. The uptake of ethidium bromide, a nucleus marker, was monitored in all experiments to evaluate cell viability and the possible contribution of cell lysis to ATP release. Cell monolayers were incubated in the presence of 0.15–0.3 μg/ml of ethidium bromide, and epifluorescence microscopy images were taken at five to eight randomly selected viewing fields of the cell monolayer at ×10 magnification. Stored images were later analyzed, and red-stained dead cells were counted.

RESULTS

Figure 2A gives an example of ATP release evoked from A549 cells by air bubble passage through the flow-through experimental chamber at 5 and 11 min. ATP content increased instantly in perfusate aliquots collected immediately after bubble passage but declined rapidly toward background during chamber perfusion. The rapid decline of ATP content in the perfusate corresponded to the rate of ATP removal from the chamber ($T_{1/2}$ = 15 s), indicating that ATP release took place only during air bubble passage and ceased immediately upon its removal from the chamber. Repeated passages of several subsequent air bubbles also produced significant responses, although they often varied significantly probably because of difficulty in controlling exact conditions of such stimulation, dwell time and air bubble size passing through the chamber.
Similar responses, although with a somewhat slower decline, were noted with human bronchial epithelial 16HBE14o− cells (Fig. 2B). The bar graphs below summarize cumulative ATP release during a 5-min period (in pmol/10⁶ cells) after air bubble stimulation. Cumulative release varied between different experiments from ~700 to ~2,400 pmol/10⁶ for A549 cells and was up to ~3,000 pmol/10⁶ for 16HBE14o− cells. The responses were significantly, up to hundredfold, above the basal release observed during similar time periods for unstimulated A549 or 16HBE14o− cells after stimulation by 6-well culture dish tilting for 30 s. The data are means ± SE from n = 6 cell monolayers. C: brief air exposure of cell monolayer by plate tilting did not cause cell damage. The data are the average number of ethidium bromide-stained dead cells (± SE) counted in 5 randomly selected viewing fields before and after plate tilting for the indicated times in experiments such as those shown in A and B.

Confocal microscopy reveals cell deformation by air bubble passage. The above experiments demonstrated that air bubble passage over the cell monolayer or culture plate tilting induced transient ATP release. Different forms of mechanical stress, including hypotonic cell swelling, are known to evoke such release from epithelial and nonepithelial cells (2). It is likely that air bubble passage or culture plate tilting may also involve some sort of mechanical stimulation. To explore this aspect in more detail, we undertook confocal microscopy cell imaging during air bubble passage and analyzed cell height. Cells were stained with 5-chloromethylfluorescein diacetate (CMFAD, 1 μg/ml), which is colorless and nonfluorescent in aqueous solution. CMFAD passes freely through cell membranes, where it is cleaved by cytosolic esterases into a brightly fluorescent product only in viable, living cells. Figure 4 shows a reconstructed x-z profile of the cell monolayer before and during air bubble passage. The experiments revealed cell deformation, flattening by ~10% up to ~40% of cell height in the monolayer part in direct contact with the air bubble, which was fully reversible after cell passage. This suggests that during air bubble passage the ALI is brought to such close proximity with the cell membrane that liquid surface tension forces may be transmitted directly onto the cells, causing their mechanical deformation.
Cell stimulation by ALI elevates [Ca\(^{2+}\)]. We have reported previously that ATP release induced by 50% hypotonic shock was tightly correlated with [Ca\(^{2+}\)]\(_i\) responses in A549 and 16HBE14o– cells (2). To investigate whether a similar relationship exists for ATP release evoked by cell deformation at the ALI, we performed ratiometric fura 2 fluorescence experiments to monitor [Ca\(^{2+}\)]\(_i\) changes during cell stimulation by air bubble passage. Figure 5A shows the [Ca\(^{2+}\)]\(_i\) response in A549 cells induced by air bubble passage, which consisted of brief <1 min [Ca\(^{2+}\)]\(_i\) spikes strikingly similar to the transient ATP release depicted in Fig. 2A. Furthermore, [Ca\(^{2+}\)]\(_i\) responses were independent of the presence of extracellular Ca\(^{2+}\), demonstrating that they do not involve nonspecific Ca\(^{2+}\) leakage from the extracellular space, e.g., due to plasma membrane damage, but could be entirely attributed to Ca\(^{2+}\) release from intracellular stores (Fig. 5B). This is similar to previously reported [Ca\(^{2+}\)]\(_i\) responses triggered by hypotonic shock in A549 cells (8, 25).

We also verified whether exposing cells to the ALI by culture dish tilting elevated [Ca\(^{2+}\)]. Since dish tilting during fura 2 microscopy imaging was impractical, we mimicked such an experiment by removing liquid covering cells in the open dish.
dish to expose them to air under ambient humidity at room temperature and short pulses of gentle air flow. Figure 5C illustrates that sustained \([\text{Ca}^{2+}]_i\) elevation occurred after a short delay. Considering the presence of the extracellular matrix, secreted proteins, etc., a thin layer of adhering liquid will be preserved on the cell surface, and it may take several minutes to slowly dry out by evaporation at room temperature and ambient humidity. As a result, for more than 15–20 min, the cells seem to be well protected by a thin liquid layer and remain viable. Except for cells at the edges of the glass coverslip, where they might be subjected to more extreme capillary forces, all cells within a monolayer showed no detectable damage after reimmersion in medium and staining dead cells with ethidium bromide (Fig. 5D). The \([\text{Ca}^{2+}]_i\) response was fully reversible and returned to baseline after covering the cells with medium.

\textbf{Ca}^{2+} \textbf{dependence of ATP release.} Next, we investigated the role of extracellular and intracellular \(\text{Ca}^{2+}\) in ATP release induced by the ALI. Removal of extracellular \(\text{Ca}^{2+}\) did not affect the time course or amplitude of air bubble-induced ATP release from A549 and 16HBE14o\textsuperscript{−} cells (Fig. 6, A and B).

Thus the ATP release studied here does not depend on \(\text{Ca}^{2+}\) influx from the extracellular space, and its short-term removal has no effect on ATP responses. In contrast, intracellular \(\text{Ca}^{2+}\) has been found in our previous study to regulate hypotonic shock-induced ATP release from lung epithelial cells (2). To investigate whether it has a similar role in mechanosensitive ATP release induced by the ALI, cells were loaded with the intracellular \(\text{Ca}^{2+}\) chelator BAPTA-AM. Figure 7, A and B, Fig. 7. Role of intracellular \(\text{Ca}^{2+}\) in ATP release. Cumulative ATP release was significantly diminished in BAPTA-loaded A549 (A) and 16HBE14o\textsuperscript{−} cells (B) stimulated by air bubble, and in 16HBE14o\textsuperscript{−} cells stimulated by plate tilting (C). Data are means ± SE from \(n = 7\) to 16 experiments; *inhibition by BAPTA was statistically significant for both cell lines (2-sample independent \(t\)-test, \(P < 0.05\); note a log scale on the y-axis).

Fig. 6. Role of extracellular \(\text{Ca}^{2+}\) in ATP release. A: time course of air bubble-induced ATP release was not affected by removal of extracellular \(\text{Ca}^{2+}\). The 2 traces are examples of such an experiment with 16HBE14o\textsuperscript{−} cells in \(\text{Ca}^{2+}\)-containing and \(\text{Ca}^{2+}\)-free extracellular solution. B: bar graph showing mean ± SE cumulative ATP release from A549 cells induced by air bubble stimulation. Cumulative release, calculated for 5-min poststimulation, was significantly elevated compared with basal release but was not different in \(\text{Ca}^{2+}\)-containing and \(\text{Ca}^{2+}\)-free extracellular solution.
demonstrates that air bubble-stimulated ATP release was significantly diminished in BAPTA-loaded A549 alveolar cells and in bronchial epithelial 16HBE14o− cells, by ~40 and ~90%, respectively, and by ~70% when 16HBE14o− cells were stimulated by plate tilting (Fig. 7C). The results reveal that ALI-induced ATP release involves a Ca^{2+}-dependent process, similar to that shown previously for ATP release induced by hypertonic shock. Also, basal release was diminished in BAPTA-loaded cells.

**NEM diminishes ATP release.** A tight correlation between ATP release and [Ca^{2+}]i suggests the involvement of Ca^{2+}-dependent exocytosis. To further strengthen this notion, we tested NEM, a sulphydryl-modifying agent that inhibits vesicular exocytosis by acting on NEM-sensitive proteins of the SNARE complex (11). Figure 8A compares the time course of ATP release from control and NEM-treated A549 cells induced by air bubble passage. ATP release was almost completely inhibited in NEM-treated cells, and the results from three such experiments are summarized in Fig. 8B.

**DISCUSSION**

Physical forces play an important role in normal lung physiology, controlling multiple processes, from gene expression and surfactant secretion in alveoli to MCC regulation in the airways (5, 14, 15). In this study, we focused on the effects of tension forces at the ALI on ATP release from lung epithelial cells. Such effects might be relevant in vivo since much of lung epithelial tissue has fluid-covered, air-exposed mucosal surfaces. The normal high surface tension of water, ~70 dyn/cm at 37°C, is greatly decreased, to ~22–25 dyn/cm, by surfactants on the alveolar surface, increasing compliance and allowing the lungs to inflate much more easily. At the end of expiration, compressed surfactant phospholipid molecules decrease surface tension even further to very low, near-zero levels (21). Interestingly, surface tension of the cell membrane is also very low, ~0.04–0.1 dyn/cm for resting cells (6), and 3.8 dyn/cm for deformed cells (20). Previous studies on T cells found that cell resistance to deformation by the ALI was not measurable. When a very thin film of liquid was drawn down on the cells, ALI forces greatly deformed them, and neither cellular surface tension nor any internal scaffolding (cytoskeleton) was able to sustain loads comparable to that induced by the ALI (20). In our experiments we used air bubble, or plate tilting, to form a thin liquid film on the apical surface of epithelial cell monolayers and exposed them to tension forces of the ALI. Any curved cell surface will be subjected to a net component of tension forces that will act perpendicularly to the cell membrane, leading to cell deformation. Its magnitude could be estimated by considering pressure P inside a liquid droplet that arises due to surface tension. For a spherical droplet of radius r this pressure is described by LaPlace's law: $P = 2\gamma/r$, where $\gamma$ is surface tension of a liquid. Taking $\gamma = ~70$ dyn/cm for pure water at 37°C and $r = 10 \mu$m gives a pressure of $1.4 \times 10^5$ dyn/cm$^2$ (equivalent of ~140 cmH$_2$O hydrostatic pressure). For a hemispherical cell or cell apex, as shown in Fig. 1A, it would be less than half of that, depending on the fraction of the cell surface exposed to tension forces. Nevertheless, compared with the typical cell elastic modulus of 1 to 10 kPa ($10^4$ to $10^5$ dyn/cm$^2$) (16), the pressure generated by surface tension is large enough to cause significant cell deformation.

Similar stress may likely act on lung epithelial cells in vivo, especially during inflation, when surfactants are dispersed on larger surface. Our confocal imaging study confirmed that A549 lung epithelial cells covered by physiological saline are indeed greatly deformed/flattened by tension forces in proximity with the ALI during air bubble passage through the imaging chamber. This mechanical deformation led to increased [Ca^{2+}]i and ATP release without cell lysis. Cumulative ATP release elicited during brief (~15-s) air bubble passage was large, ~1,000 pmol/10$^6$ cells (Fig. 2B), comparable to 15-min cumulative release induced by 50% hypertonic shock (2). Similar release was observed when cells were stimulated by air exposure during culture plate tilting. The amount of ATP corresponds to the total releasable ATP pool in A549 cells, i.e., the amount of ATP that could be released by hypertonic shock, which was ~1,000–1,600 pmol/10$^6$ cells, or ~5% of cellular ATP (2, 25). This suggests that 15-s air bubble stimulation almost entirely depletes the releasable pool of ATP in A549 cells, which is quickly replenished since after ~5–10 min, the cells could respond to repeated stimulation.

It is interesting that ALI-induced ATP release from A549 and 16HBE14o− cells shares many similarities with that evoked by 50% hypertonic shock. In both cases, ATP release was tightly correlated with [Ca^{2+}]i responses but was independent of extracellular Ca$^{2+}$. This demonstrates that the [Ca$^{2+}$i] response could be entirely attributed to Ca$^{2+}$ release from...
intracellular stores and that it does not imply nonspecific Ca\(^{2+}\) leakage from the extracellular space, e.g., due to plasma membrane damage. The ATP response was, however, strongly modulated by [Ca\(^{2+}\)], as validated by significantly reduced ATP release from BAPTA-loaded cells, which is consistent with a release mechanism involving regulated, Ca\(^{2+}\)-dependent exocytosis. This notion was further confirmed in experiments with NEM, a sulfhydryl-modifying agent commonly used to inhibit vesicular exocytosis. NEM treatment almost completely abolished ATP release evoked by the ALI (Fig. 8). These findings exactly mirror those reported for hypotonic shock-induced ATP release from A549 cells (2, 25). Consistent with Ca\(^{2+}\)-dependent exocytotic release the previous study also revealed enhanced [Ca\(^{2+}\)]\(_i\) response and autoamplification of nucleotide release due to autocrine/paracrine action of extracellular nucleotides on cell surface P2Y receptors in A549 cells. Because autocrine/paracrine purinergic loop will be triggered regardless of the stimuli that initiates nucleotide release, cells. Because autocrine/paracrine loop will be triggered regardless of the stimuli that initiates nucleotide release, it likely contributes to ATP and [Ca\(^{2+}\)]\(_i\) responses evoked by tension forces in the present investigations.

Our past observation that removing culture media while tilting the plate causes massive ATP release (9) could now be explained by cell deformation elicited by tension forces when cells are exposed to the ALI during such manipulations. Several factors affect formation of a thin liquid film on an inclined plane, including surface roughness and wetting forces, liquid viscosity, surface tension, and surface tension gradients (17). Furthermore, this phenomenon is strongly affected by ambient conditions: air flow, temperature, and humidity. For example, no ATP release was detected, even after prolonged tilting (~30 min) when the culture dish was placed inside a humidified incubator, consistent with the fact that cell stimulation by tension forces at the ALI requires the formation of a sufficiently thin liquid layer covering the cells. Such a condition is facilitated by evaporation under air flow, e.g., under the hood during normal cell manipulations, such as media change, or while transferring the coverslip with cells from the culture dish to the experimental chamber (9, 10). Such unintentional cell exposure to ALI during standard cell manipulations, even in the absence of cell damage, should be avoided since the resulting ATP release may potentially affect the set point of signal transduction pathways (19).

One of the most important observations from our present study is that, under controlled conditions, ALI-induced ATP release does not involve cell lysis. The increase in average number of ethidium bromide-stained dead cells was less than ~0.5–1% after ALI exposure, indicating that contribution of ATP originating from damaged cells amounts to less than 2–8% of cumulative ATP release observed in these experiments. Thus in this study, [Ca\(^{2+}\)]\(_i\) elevation and ATP release induced by ALI forces are cellular responses that do not involve plasma membrane damage. Such responses may have physiological roles in vivo, e.g., in controlling surfactant, mucin, and fluid secretion in the lungs. Certain cell types might be predisposed to sensing proximity to the ALI, e.g., by physical location at the tips of airway folds, or by shape. Cuboidal type II cells, for instance, are more prone to ALI exposure and deformation than flat, squamous type I cells and may function as mechanotransducers for these stimuli. Surfactant secretion by type II cells is stimulated by lung expansion and involves intercellular spreading of [Ca\(^{2+}\)]\(_i\) oscillations in type II and type I alveolar cells, the latter likely acting as mechanotransducers responding to cell stretch (1). Our present study suggests that mechanosensitive ALI-induced nucleotide release may also contribute to Ca\(^{2+}\)-dependent surfactant secretion processes by activating P2Y\(_2\) cell surface purinoreceptors. In addition, we have recently shown that hypotonic shock-induced ATP release from alveolar A549 cells is associated with exocytotic corelease of several species of adenosine and uridine nucleotides (26) and that ATP release is strongly, up to threefold, amplified by autocrine effects of coreleased UDP acting on P2Y\(_6\) purinoreceptors (25). Such a synergistic action of coreleased nucleotides may have important roles in amplification and propagation of cytosolic [Ca\(^{2+}\)] responses in alveoli, supporting Ca\(^{2+}\)-dependent lamellar body fusion, pore opening, and surfactant secretion processes.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise are declared by the author(s).

**REFERENCES**


