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Paracrine stimulation of surfactant secretion by extracellular ATP in response to mechanical deformation

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Patel, Anand S., David Reigada, Claire H. Mitchell, Sandra R. Bates, Susan S. Margulies, and Michael Koval. Paracrine stimulation of surfactant secretion by extracellular ATP in response to mechanical deformation. *Am J Physiol Lung Cell Mol Physiol* 289: L489–L496, 2005. First published May 20, 2005; doi:10.1152/ajplung.00074.2005.—We developed a heterologous system to study the effect of mechanical deformation on alveolar epithelial cells. First, isolated primary rat alveolar type II (ATII) cells were plated onto silastic substrata coated with fibronectin and maintained in culture under conditions where they become alveolar type I-like (ATI) cells. This was followed by a second set of ATII cells labeled with the nontransferable, vital fluorescent stain 5-chloromethylfluorescein diacetate to distinguish them from ATI cells. By morphometric analysis, equibiaxial deformation (stretch) of the silastic substratum induced comparable changes in cell surface area for both ATII and ATI cells. Surfactant lipid secretion was measured using cells metabolically labeled with [^{3}H]choline. In response to 21% tonic stretch for 15 min, ATII cells seeded with ATI cells secreted nearly threefold more surfactant lipid compared with ATII cells seeded alone. ATI cells did not secrete lipid in response to stretch. The enhanced lipid secretion by ATII plus ATI cocultures was inhibited by treatment with apyrase and adenosine deaminase, suggesting that ATP release by ATI cells enhanced surfactant lipid secretion at 21% stretch. This was confirmed using a luciferase assay where, in response to 21% stretch, ATI cells released fourfold more ATP than ATII cells. Because ATI cells release significantly more ATP at a lower level of stretch than ATII cells, this supports the hypothesis that ATI cells are mechanosensors in the lung and that paracrine stimulation of ATII cells by extracellular ATP released from ATI cells plays a role in regulating surfactant secretion.

pulmonary surfactant; alveolar epithelia; phosphatidylcholine; pneumocytes; adenosine 5'-triphosphate

TYPE II ALVEOLAR EPITHELIAL CELLS secrete pulmonary surfactant in response to mechanical deformation (26, 59). However, the adult alveolar epithelium is a mixed monolayer consisting of type I and type II cells. Although type II cells outnumber type I cells by a 2:1 ratio in the normal lung, >90% of the alveolar surface is covered by large, flat type I cells that serve as the surface for gas exchange with the external environment (18). Virtually all type II cells are in direct contact with type I cells, suggesting that the type II-type I cell interface may have a role in regulating alveolar function (39).

Using in situ fluorescence microscopy, Ashino et al. (3) demonstrated that type I cells serve as mechanosensors in the lung. In response to hyperinflation, a transient increase in cytosolic calcium was initiated by type I cells, which was then transmitted to type II cells through gap junctions. This in turn stimulated lamellar body-plasma membrane fusion by type II cells, as measured by release of LysoTracker dye preloaded into the lamellar bodies. Consistent with this, mixed cultures of isolated alveolar epithelial cells with type II-like (ATII) and type I-like (ATI) phenotypes form functional gap junctions with the capacity to transmit mechanically initiated calcium transients from one cell type to another (2, 33, 36). On the other hand, calcium transients can also be transmitted to ATII cells by extracellular ATP through subsequent paracrine stimulation of purinergic receptors (37).

ATP is a potent secretagogue that stimulates type II cell surfactant secretion (22, 27, 41, 49). Pharmacological and molecular evidence supports the expression of $P2Y_2$ receptors by type II cells (27, 48). However, alveolar epithelial cells also express P2X-type purinergic receptors (45, 60). In addition to ATP, agents that stimulate cAMP formation, including adenosine and adenosine analogs, enhance surfactant secretion through PKA-dependent pathways (29, 32). Although secretion of surfactant lipid by type II cells in response to purines and other agonists has been pharmacologically well characterized (49), little is known about the origin of extracellular ATP in the alveolus.

To date, the mechanical stimulation of surfactant lipid secretion by ATII cells in vitro has been examined only in monocultures. To mimic elements of a native alveolar epithelium, we used a coculture model based on two separate isolations of rat alveolar epithelial cells. The first preparation consisted of alveolar epithelial cells cultured under conditions where they lose type II cell markers (e.g., surfactant proteins) and gain expression of type I cell markers (including T1α and aquaporin-5), which we refer to as ATI cells. Although ATI cells generated from cultured ATII cells are not bona fide type I cells (31), they are a practical model system that expresses several hundred genes in common with type I cells and that has similar morphology (10–12, 19, 31, 39, 43, 45, 55, 58). The second preparation consisted of freshly isolated ATII cells fluorescently labeled to distinguish them from the ATI cells. This second isolation was seeded onto the ATI monolayer to create an alveolar epithelial coculture model. We found that surfactant lipid secretion by ATII cells cocultured with ATI cells was more sensitive to stretch than ATII cells alone. This was due to stretch-stimulated ATP release by ATI cells and suggests a paracrine pathway to stimulate surfactant release.

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METHODS

Cell culture. Animal protocols were reviewed and authorized by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Sprague-Dawley rat ATII cells were isolated from lavaged, perfused lungs by elastase digestion using the method of Dobbs et al. (23) with modifications (1). With the use of this approach, preparations routinely contained 90–95% ATII cells (1). Cells were cultured in Earle's MEM (Life Technologies, Rockville, MD) containing 10% FBS, 25 μg/ml gentamicin, and 0.25 μg/ml amphotericin B (Life Technologies) at a seeding density of 1×10^6 cells/cm^2 on either tissue culture-treated plastic or on custom-made wells containing a deformable silastic substratum coated with 10 μg/cm^2 of fibronectin (Boehringer Mannheim Biochemicals) as previously described (55). Cell attachment was limited to the central portion of each well by placing a piece of Tygon tubing in the center of each well and seeding cells only within this restricted area. Cells were studied *1*) after overnight culture (ATII) or *2*) after 5–6 days (ATI cells) or *3*) the ATI cells were used for coculture experiments. For cocultures, a second preparation of ATII cells was made using the protocol described above except isolated ATII cells in suspension were labeled with medium containing 10 μM CellTracker Green 5-chloromethylfluorescein diacetate (CMFDA; Invitrogen-Molecular Probes, Eugene, OR). Under these conditions, CMFDA permeates into the cells and covalently binds to proteins, providing a vital, nontransferable fluorescent stain that remains specifically associated with the second set of ATII cells (44). CMFDA-labeled ATII cells were cultured alone or were cultured on plates or substrata containing preexisting ATI cells (plating density = 1×10^6 cells/cm^2). Using FITC-anti-cytokeratin (Sigma) as an epithelial cell marker and Cy3-anti-vimentin (Sigma) as a fibroblast marker, we found that ATI cell preparations contained 86.5 ± 7.9% epithelial cells ($n = 6$ fields, 2 independent preparations). Expression of type I cell markers by these ATI cells was previously demonstrated (55).

Quantitative fluorescence and microscopy. To determine whether cell-associated CMFDA fluorescence was a linear function of cell number, a specific number of ATII cells was plated into a multiwell tissue culture dish and cultured overnight, and then CMFDA fluorescence was measured using a PE Biosystems microplate fluorometer (excitation: 485 nm, emission: 535 nm). The relative fluorescence units (RFU) were correlated to the input number of ATII cells. A similar method was used to quantify ATII cell number in cocultures stimulated with ATP. For cocultures on silastic substrata, cells were imaged by phase-contrast and fluorescence microscopy using an Olympus IX70 with a U-MWIBA filter pack (BP460–490, DM505, BA515-550). Images were acquired using Image Pro software and threshold filtered to select areas with fluorescent cells, which were then quantified to give the fraction of the surface covered by CMFDA-labeled cells.

Changes in cell area in response to mechanical deformation were determined by morphometric analysis. Cells were cultured on silastic substrata contained in a plastic well device that allowed equibiaxial mechanical stretch of the substratum (55) (see Fig. 3). The top piece of the device was mounted into a threaded holder and manually turned to lower the substratum onto a hollow ring-shaped indentor. More rotations lowered the substratum a greater amount onto the indentor; in other words, more turns resulted in greater stretch of the substratum. Cells in coculture were imaged by phase-contrast and fluorescence microscopy, and the change in cell surface area as a function of number of turns was determined using Image Pro by manually tracing cell outlines and morphometric analysis of cell area.

Assay for secreted surfactant lipid. Cells were incubated overnight in medium containing 0.5 mCi [^{3}H]choline chloride. The cells were then washed, the medium was replaced, and the cells were incubated at 37°C for a 30-min recovery period. An aliquot of medium was removed as a baseline control, and then surfactant secretion was stimulated by adding ATP from a 100× concentrated stock (1 mM final concentration) followed by a 2-h incubation at 37°C, conditions previously optimized for ATP-stimulated surfactant lipid release (5, 28). For mechanical deformation, cells were stretched by the indicated amount for 15 min and then relaxed and further incubated at 37°C for 2 h total (59). Unstimulated cells in parallel cultures were used as controls. Lipids were isolated from medium and cell fractions by Bligh-Dyer extraction and measured by liquid scintillation counting as previously described (5). The amount of surfactant [^{3}H]phosphatidylcholine ([^{3}H]PC) in the medium was normalized to total radiolabeled lipid (medium + cells) or to CMFDA fluorescence as determined above.

To inhibit receptor stimulation by secreted ATP, cells were preincubated for 30 min and mechanically stimulated in the presence of a mixture of 20 U/ml apyrase (A-6535; Sigma, St. Louis, MO) and 5 U/ml adenosine deaminase (ADA; A-5168, Sigma) to metabolize ATP and adenosine to an inactive form (inosine). Some experiments were done using cells treated with apyrase alone. To inhibit gap junctional communication, cells were pretreated for 30 min and mechanically stimulated in the presence of either a mixture of connexin mimetic peptides (160 μM GAP-26 + 130 μM GAP-27) (24) or 40 μM β-glycyrrhetinic acid (20). To measure gap junctional communication between ATI cells, a glass micropipette containing 2 mg/ml of Alexa Fluor 488 (Molecular Probes) in water was used to microinject a single cell in a field, and the diffusion of Alexa 488 by gap junctional intercellular communication was assessed as the number of cells containing fluorescent dye after a 3-min incubation period (40). A cell was scored as positive if it had a representative area with an average fluorescence intensity of at least 10% of the microinjected cell fluorescence intensity as determined with Image Pro. ATI-ATII cell communication was measured using the preloading assay as previously described (2).

ATP concentration measurements. ATII cells alone or ATI cells alone on deformable substrata were washed and the culture medium was replaced with 2 ml of serum-free Earle's MEM containing 100 μM ARL-67156 to inhibit extracellular ATP degradation by ectoenzymes (17, 38). A 50-μl aliquot of medium was harvested, frozen, and stored at −20°C. The cells were then stretched by the indicated amount for 10 min, and a second 50-μl aliquot of medium was harvested and frozen. Care was taken to avoid disrupting the cell monolayer while obtaining aliquots of medium for the ATP assay. Medium ATP concentration was detected with the chemiluminescent luciferin-luciferase assay using previously published techniques (47). In brief, luciferin-luciferase stock solution was prepared from one vial of ATP assay solution (Sigma) plus 450 μl of isotonic buffer (105 mM NaCl, 5 mM KCl, 10 mM HEPES, 5 mM $NaHCO_3$, 60 mM mannitol, 5 mM glucose, 0.5 mM $MgCl_2$, 1.3 mM $CaCl_2$, pH 7.4) plus 50 μl of distilled water. This stock solution was further diluted 25-fold in Earle's MEM to make a working luciferin-luciferase assay solution. Twenty-five microliters of each medium sample were placed into a well of an opaque white 96-well plate and diluted with an equal volume of luciferin-luciferase assay solution. Light emitted from luciferase luminescence, which is proportional to the concentration of ATP in the solution, was recorded with a Luminoskan Ascent microplate luminometer (Labsystems, Franklin, MA). Luminescence measurements were taken every minute for 15 min with an integration time of 100 ms/well. The ATP content in each sample was calculated as the mean signal obtained in the first 5 min of measurements calibrated to a standard curve.

Statistics. Data are presented as means ± SE. Least squares analysis was calculated with Microsoft Excel, and statistical significance was calculated with SigmaStat (Jandel Scientific) using either a standard *t*-test or paired *t*-test as noted in the figure legends. For comparisons, P values <0.05 were considered to reflect statistically significant differences.

RESULTS

We used a model based on two preparations of isolated rat alveolar epithelial cells as a method to mimic interactions that occur between alveolar epithelial cells. To distinguish between the two different cell types in coculture, CellTracker CMFDA was used as a vital fluorescent stain to label a subpopulation of alveolar epithelial cells. As shown in Fig. 1, CMFDA incorporation by ATII cells was a linear function of cell number. CMFDA remained associated with ATII cells after overnight incubation and did not transfer to unlabeled ATI cells. By phase-contrast microscopy, CMFDA-labeled cells showed lamellar bodies suggesting they have the capacity to secrete surfactant lipid. Also, cocultures contained 1.2 ± 0.4 (n = 8) ATII cells for each ATI cell, slightly less than the 2:1 ratio typically found in the adult lung (18).

Surfactant lipids in ATII cells can be metabolically labeled using [^{3}H]choline to produce [^{3}H]PC. With the use of this approach, it was found that extracellular ATP stimulates isolated ATII cells to secrete [^{3}H]PC (5, 28). We confirmed that metabolically labeled ATII cells incubated for 2 h with 1 mM ATP were stimulated to secrete [^{3}H]PC compared with unstimulated controls (Fig. 2*A*). In contrast, ATI cells secreted little, if any, [^{3}H]PC in response to extracellular ATP, consistent with a previous report (53). This also suggests that in cocultures, any [^{3}H]PC secreted would be due to ATII cells rather than ATI cells.

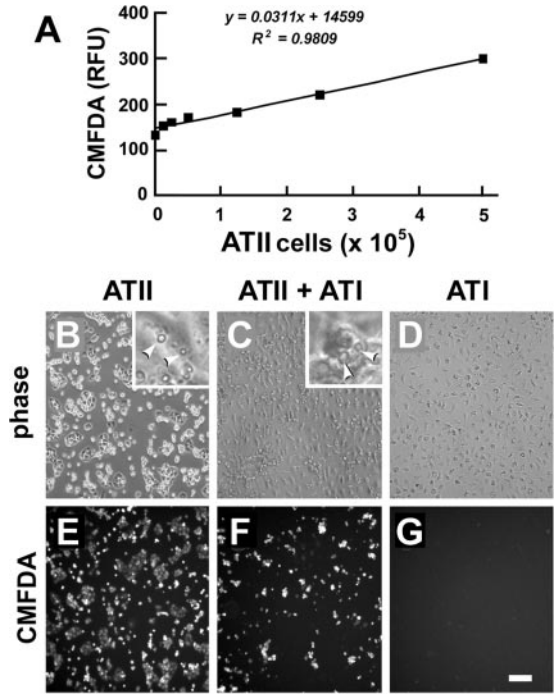

Fig. 1. Vital cell staining of alveolar epithelial cells is shown. *A*: alveolar type II (ATII) cells were isolated and labeled with the fluorescent vital dye 5-chloromethylfluorescein diacetate (CMFDA) during the biopanning step of the isolation procedure, seeded to different densities, and cultured overnight in a 24-well dish. Cell-associated fluorescence was measured using a PE Biosystems fluorescence microplate reader. Data points represent the means of triplicate wells, and SD was less than data point symbol size. By least squares analysis, cell-associated CMFDA relative fluorescence units (RFU) were linearly related to ATII cell number. *B–G*: cultures containing only CMFDA-labeled ATII cells (*B* and *E*), a mixture of ATII and alveolar type I-like (ATI) cells (*C* and *F*), or ATI cells alone (*D* and *G*) were visualized by phase-contrast microscopy (*B–D*) and labeled ATII cells by CMDFA fluorescence (*E–G*). *Insets* show 7-fold magnified phase-contrast images of ATII cells containing lamellar bodies (arrowheads). Bar, 100 μm.

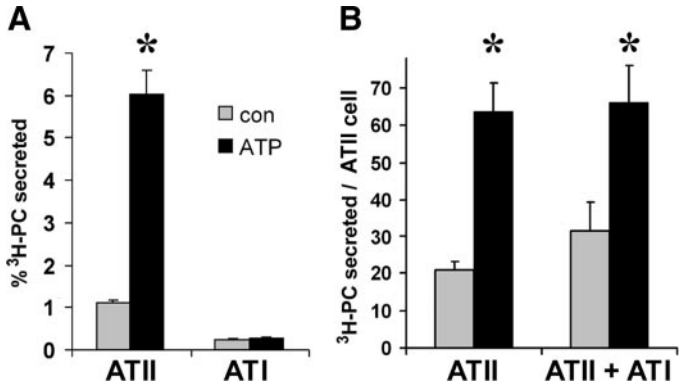

Fig. 2. ATP-stimulated phospholipid secretion by ATII cells was comparable for monocultures and cocultures. *A*: cells were metabolically labeled overnight with [^{3}H]choline, washed, and then treated with 1 mM ATP for 2 h at 37°C to stimulate [^{3}H]phosphatidylcholine ([^{3}H]PC) secretion. Shown is the mean percentage ± SE of total [^{3}H]PC secreted into the medium by unstimulated [control (con), gray bars] or ATP-stimulated (black bars) cells. Monocultures of ATII cells showed significant [^{3}H]PC secretion in response to ATP compared with untreated controls; however, ATI cells were not responsive to ATP. *B*: CMFDA-labeled ATII cells alone or cocultured with ATI cells were metabolically labeled overnight and stimulated with ATP as above. The amount of [^{3}H]PC secreted into the medium by unstimulated (con, gray bars) or ATP-stimulated cells (black bars) was normalized to CMFDA fluorescence ([^{3}H]PC secreted/ATII cells) as described in METHODS. Data were calculated from 3 independent determinations; *significantly different from unstimulated controls ($P < 0.05$).

Metabolically labeled lipid provides a direct measure of surfactant secretion. This method works well for isolated ATII cells, since the amount of secreted lipid can be normalized to the total lipid pool as a way to control for the number of ATII cells per dish. However, when ATII cells are combined with ATI cells, [^{3}H]choline is incorporated into the intracellular PC pools of both ATII and ATI cells. Thus to compare the amount of surfactant lipid secreted by ATII cells alone to the amount of secretion by ATII cells cocultured with ATI cells, we normalized the amount of [^{3}H]PC secreted to the number of ATII cells using CMFDA fluorescence ([^{3}H]PC secreted/ATII cell). CMFDA incorporation by ATII cells was linear with respect to ATII cell number, and ATI cells were not fluorescent. Using this approach, we found that ATII cells were stimulated to secrete comparable amounts of [^{3}H]PC in response to 1 mM ATP regardless of whether they were alone or combined with ATI cells (Fig. 2*B*). Thus ATI cells did not alter the ability of ATII cells to secrete surfactant lipid in response to extracellular ATP.

To assess the effect of stretch on alveolar epithelial cells, we used a previously described cell deformation apparatus (55). Cells were plated onto a fibronectin-coated silastic substratum in a well mounted into a holder/indentor (Fig. 3*A*). Cells were stretched by rotating the well a defined number of turns, which caused the indentor to push up on the substratum. This in turn stretched the substratum, creating an equibiaxial deformation (55). Microscopy and morphometric analysis were used to measure changes in cell surface area as a function of the number of turns for ATI and ATII cells in coculture. Both ATI and ATII cells had comparable changes in cell size as a function of number of turns. Thus the ATII cells were firmly

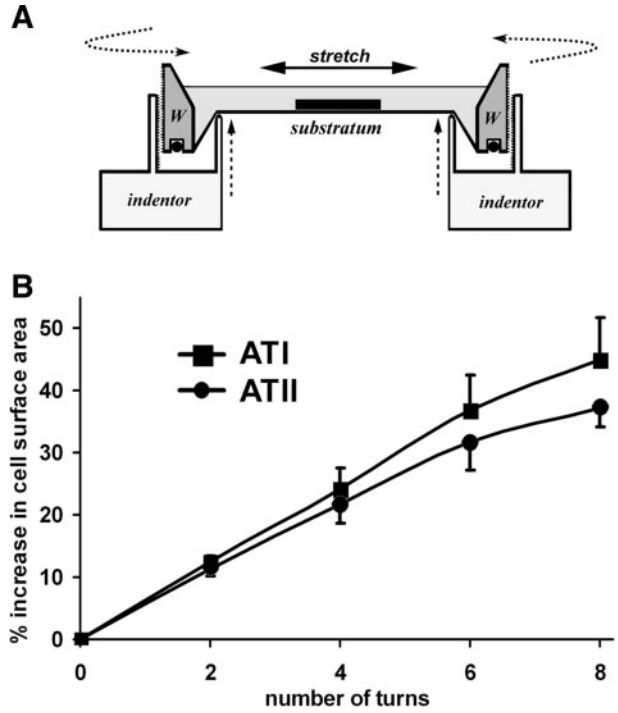

Fig. 3. Mechanical deformation of ATII and ATI cells is depicted. *A*: cross-section of a well designed for mechanical deformation of cells. W, well; black rectangle denotes the area of the substratum where the cells are adherent. The silastic substratum is stretched by turning the well, which causes the indentor to push up onto the substratum (55). *B*: CMFDA-labeled ATII cells and unlabeled ATI cells cocultured on a silastic substratum were progressively stretched. Shown is mean cell area ± SE calculated morphometrically as a function of the number of turns of the well in the indentor housing. Data are from 5 independent wells, measuring 5–8 cells of each type per well. Both ATII cells (●) and ATI cells (■) increased in size with increasing number of turns.

attached to the ATI cells and/or the extracellular matrix on the silastic substratum.

Wirtz and Dobbs (59) previously found that metabolically labeled ATII cells secrete [^{3}H]PC in response to mechanical deformation. As shown in Fig. 4, we confirmed that ATII cells mechanically stretched to 25% were stimulated to secrete [^{3}H]PC. However, ATI cells did not secrete [^{3}H]PC in response to stretch. As was the case for ATP-stimulated [^{3}H]PC secretion, this suggests that any [^{3}H]PC secreted into the medium by cocultures in response to mechanical stimulation would be from ATII cells, not ATI cells.

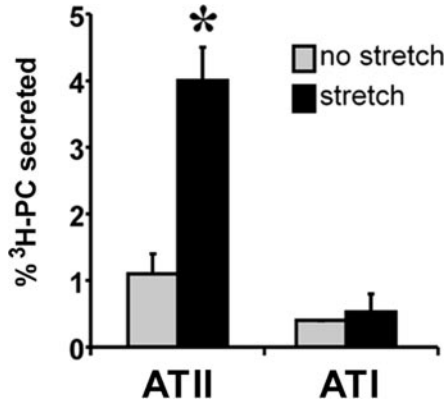

Fig. 4. ATI cells do not secrete PC in response to mechanical deformation. ATII cells alone or ATI cells alone were labeled overnight with [^{3}H]choline, washed, tonically stretched to 25% for 15 min, released, and then further incubated for 2 h at 37°C. Shown is the mean percentage ± SE of total [^{3}H]PC secreted into the medium by unstimulated (con, gray bars) or stretched (black bars) cells. Data were calculated from 3 independent determinations; *significantly different from unstimulated controls ($P < 0.05$).

[^{3}H]PC secretion by ATII cells alone or cocultured with ATI cells was determined as a function of increasing levels of mechanical deformation (Fig. 5). For these experiments, the level of [^{3}H]PC secreted/ATII cell RFU was calculated so that unstretched controls were set to a value of 0. For each magnitude of stretch tested, cells were stimulated to secrete more [^{3}H]PC than unstretched controls. At 12% stretch, ATII cells secreted comparable levels of [^{3}H]PC regardless of whether or not ATI cells were present. However, at 21% stretch, there was a difference in the level of surfactant lipid secretion. ATII cells cocultured with ATI cells secreted roughly two- to threefold more [^{3}H]PC than ATII cells alone. Thus coculture with ATI cells enhanced the ability of the ATII cells to respond to 21% stretch. At 30% stretch, ATII cells cocultured with ATI cells maintained this higher level of [^{3}H]PC secretion. ATII cells alone also showed a higher level of [^{3}H]PC secretion at 30% compared with lesser stretch magnitudes. This is consistent with a fluorescence microscopy study that showed that ATII cells had significantly more lamellar body-plasma membrane fusion at 30–50% stretch as opposed to 10–20% stretch where few lamellar body fusion events were detected (26). Thus the amount of [^{3}H]PC secreted in response to mechanical deformation was a function of both cell population and the magnitude of stretch. In particular, the enhanced amount of [^{3}H]PC secreted by ATII cells cocultured with ATI cells at 21% stretch indicated that there was likely to be signaling from ATI cells to ATII cells.

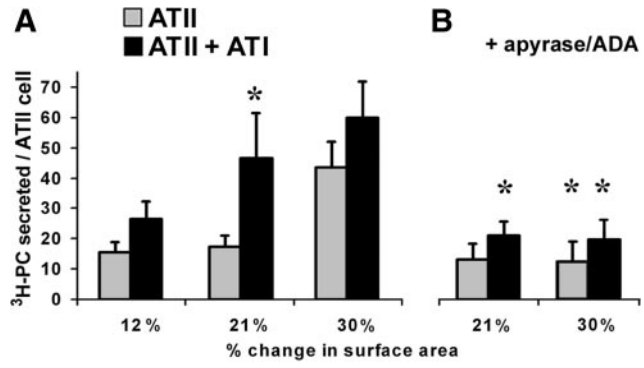

Fig. 5. ATII cells cocultured with ATI cells secrete surfactant lipid at a lower mechanical threshold than ATII monocultures. *A*: CMFDA-labeled ATII cells alone (gray bars) or cocultured with ATI cells (black bars) were tonically stretched for 15 min by either a 12, 21, or 30% increase in ATII cell area and were then released and further incubated for 2 h at 37°C. [^{3}H]PC secretion was measured and normalized to the number of ATII cells using CMFDA fluorescence ([^{3}H]PC secreted/ATII cells) as described in METHODS. Data are the means ± SE of 8 independent sets of duplicate determinations with background corrected for secretion by unstretched controls. There was significantly more [^{3}H]PC secreted by cocultures at 21% stretch than monocultures (*$P <$ 0.05 by paired Student's *t*-test). *B*: CMFDA-labeled ATII cells in monoculture (gray bars) or cocultured with ATI cells (black bars) were pretreated with apyrase and adenosine deaminase (ADA) for 30 min, tonically stretched for 15 min at 21 or 30%, further incubated for 2 h at 37°C in the presence of the enzymes, and analyzed as in *A*. *Significantly different from stretched cells in the absence of enzymes (*A*); $P < 0.05$ by paired Student's *t*-test.

To determine whether gap junctional communication contributed to the increased [^{3}H]PC secretion by ATII cells plus ATI cells at 21% stretch, we used two different gap junction inhibitors known to inhibit gap junctional communication between alveolar epithelial cells: either a mixture of connexin mimetic peptides (160 μM GAP-26 + 130 μM GAP-27) (9, 24) or β-glycyrrhetinic acid (1, 20). Neither treatment had a significant effect on surfactant lipid secretion at 21% stretch. Cocultures pretreated and stretched in the presence of GAP inhibitor peptides secreted 96.9 ± 0.7% of the level of [^{3}H]PC secreted by untreated control cocultures. Also, cocultures pretreated and stretched in the presence of 40 μM β-glycyrrhetinic acid secreted 107.6 ± 2.4% of the level of [^{3}H]PC secreted by untreated control cocultures. Thus we were unable to detect a role for gap junctional communication in the control of surfactant secretion in this experimental system. Despite the lack of an affect on [^{3}H]PC secretion, β-glycyrrhetinic acid inhibited gap junctional communication between ATI cells by 95%, whereas control cells showed extensive transfer of microinjected Alexa Fluor 488 [16.5 ± 2.3 labeled cells/injection (n = 12)] vs. β-glycyrrhetinic acid-treated cells [0.8 ± 0.8 labeled cells/injection (n = 7)]. Qualitatively, β-glycyrrhetinic acid also inhibited gap junctional communication between ATI and ATII cells, as measured using the preloading assay. Although gap junction inhibitors did not inhibit stretch-induced [^{3}H]PC secretion, it is important to note that, even in coculture with ATI cells, ATII cells are also directly stretched, which was likely to influence their response (see DISCUSSION).

To determine whether extracellular ATP release contributed to the increased sensitivity of cocultures to mechanical stimulation, ATII cells alone or cocultured with ATI cells were pretreated with a combination of apyrase and ADA to completely metabolize any extracellular ATP released by cells in response to mechanical deformation. Although the main end product of apyrase is AMP, ADA was included to degrade any potential adenosine generated by AMP hydrolysis, since adenosine is known to stimulate surfactant secretion (29, 32). In each case, cells were first pretreated with ATPases and then mechanically stimulated in the presence of the enzymes. At 21% stretch, ATPases had little effect on ATII cells alone (Fig. 5*B*). In contrast, at 21% stretch, ATPases significantly inhibited [^{3}H]PC secretion by ATII cells cocultured with ATI cells. At 30% stretch, ATPases inhibited surfactant secretion by ATII cells regardless of whether they were alone or cocultured with ATI cells. Thus, at high levels of mechanical deformation, there was a component of [^{3}H]PC secretion due to extracellular ATP release, regardless of whether ATI cells were present. This was further confirmed to be due to extracellular ATP release, since apyrase alone inhibited stretch-induced [^{3}H]PC secretion by ATII cells [73 ± 24% inhibition (n = 2)] and cocultures [60 ± 13% (n = 2)] to levels comparable to cells treated with both apyrase and ADA (Fig. 5).

To directly measure ATP release by mechanically stimulated cells, we used a luciferase assay to measure ATP secreted into the medium by either ATI or ATII cells. Note that our measurements of ATP release were taken using cells treated with ARL-67156 to inhibit degradation of extracellular ATP by ecto-ATPases (17, 38). As shown in Fig. 6, ATI cells showed significantly more ATP release at 21% stretch than ATII cells. At 30% stretch, both ATI and ATII cells released ATP. Comparable results were obtained at 5 and 10 min following

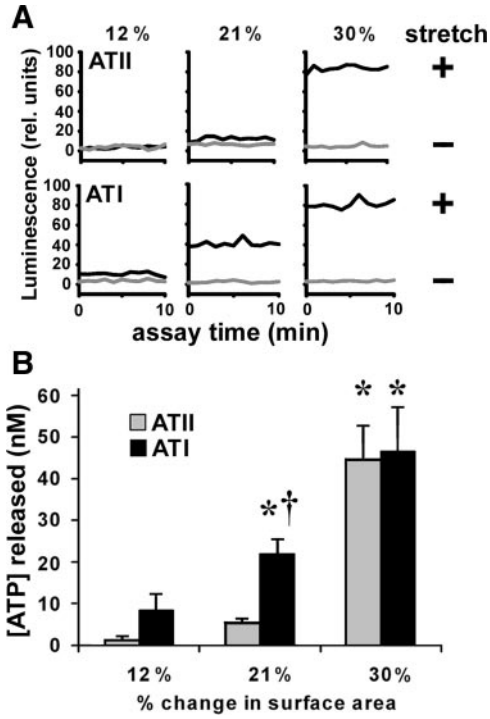

Fig. 6. ATI cells release ATP at a lower mechanical threshold than ATII cells. *A*: representative luminometric traces showing medium ATP concentration for ATII cells (*top*) or ATI cells (*bottom*). Gray lines denote readings from medium harvested before stretch (−); black lines denote medium harvested after 10 min of stretch at either 12, 21, or 30% increase in cell area (+). Readings collected during the first 5 min of collecting luminescence data were averaged to provide a measure of luminescence from each individual sample. *B*: luminescence measurements were calibrated to an ATP standard curve. Bars represent means ± SE for n = 3–4 independent determinations for ATII cells (gray bars) or ATI cells (black bars) following 10 min of stretch at 12, 21, or 30% increase in cell area. ATP release by ATI cells was significantly greater than unstretched controls for 21 and 31% stretch and by ATII cells for 30% stretch (*$P < 0.05$). ATI cells also were significantly more sensitive than ATII cells to release ATP at 21% stretch (†$P < 0.05$).

stretch, suggesting that stretch-induced ATP release had reached a plateau value. For instance, medium collected from ATI cells 5 and 10 min after initiating 21% stretch contained 23.6 ± 12.8 nM ATP and 22.0 ± 3.6 nM ATP, respectively (n = 4). These results were consistent with the results in Fig. 5 and suggest that ATP release by ATI cells plays a role in mechanically stimulated surfactant secretion by ATII cells.

DISCUSSION

In response to mechanical deformation, ATII cells secrete pulmonary surfactant (59). We found that at 21% stretch, surfactant lipid secretion by ATII cells was stimulated by paracrine ATP release by ATI cells. At higher levels of stretch, ATII cells also showed an autocrine ATP stimulated surfactant secretion pathway. This suggests that mechanically stimulated extracellular ATP release might be a normal function of type I cells to provide a pathway for mechanically stimulated surfactant release at modest levels of stretch.

Consistent with the notion of a specific ATP release pathway for alveolar epithelial cells, other types of epithelial cells have been shown to mediate physiological secretion of ATP in response to mechanical or osmotic stress (8, 51). There are several distinct mechanisms that have been implicated in ATP

release, including plasma membrane channels, such as cystic fibrosis transmembrane conductance regulator (CFTR) or a CFTR-regulated channel, other ATP-binding cassette transporters such as P-glycoprotein, maxi-anion channels, organic anion transporters, and connexin hemichannels (reviewed in Ref. 51). Many of these channels are present in alveolar epithelial cells, including CFTR (14) and P-glycoprotein (15). Although the mechanisms mediating mechanically induced ATP release by alveolar epithelial cells remain to be determined, we can rule out the possibility of ATP permeability through plasma membrane gap junction hemichannels (4, 16, 52), since gap junction inhibitors had little effect on enhanced surfactant lipid secretion by cocultures subjected to 21% stretch.

Differences in mechanical sensitivity were not likely to be due to a difference in cell adhesion, since ATI and ATII cells were well attached to the membrane whether or not they were in monocultures or cocultures, and their deformation was indistinguishable from the membrane substrate (Fig. 3 and Ref. 54). One possibility is that a difference in mechanical sensitivity was due to the greater surface area for ATI cells compared with ATII cells, a trait ATI cells have in common with type I cells in situ. ATI cells have a large, flat surface in contact with the substratum with the potential to have a higher capacity to transduce changes in substratum surface area than the more cuboidal ATII cells. Also, ATI and ATII cells have differences in cytoskeletal organization as determined using beads coated with Arg-Gly-Asp (RGD) peptides (RGD beads) that bind to integrins at the cell surface and magnetic twisting cytometry to measure the extent of integrin association with the cytoskeleton (7). Berrios et al. (7) showed that RGD beads bound to ATI cells had twice the resistance to rotation than RGD beads bound to ATII cells. This suggests that integrins expressed by ATI were more tightly associated with the cytoskeleton than ATII cell integrins. Because integrin-cytoskeletal interactions are a critical component of stretch-induced signal transduction (34), a more stable integrin-cytoskeleton complex for ATI cells compared with ATII cells is consistent with the notion that ATI cells were more efficient at transducing mechanical signals than ATII cells.

Critically, we can rule out plasma membrane rupture as a mechanism for ATP release by ATI cells. Tschumperlin and colleagues (55, 56) have shown that ATI cells are remarkably resistant to stretch-induced injury compared with ATII cells, even at extreme levels of stretch beyond the magnitudes tested here. As previously determined (55), 21% tonic stretch causes plasma membrane rupture in roughly 13% of ATII cells and $<1\%$ of ATI cells. However, at the same stretch magnitude, ATI cells secreted approximately fivefold more ATP than ATII cells (Fig. 6). Thus ATI cells were less susceptible to damage than ATII cells yet secreted more ATP. ATI cell damage is also extremely low at 30% stretch (55). Whether ATII cell damage plays a role in ATP release at 30% stretch where roughly 30% of ATII cells are damaged remains to be determined. However, we can rule out cell damage as a source for enhanced [^{3}H]PC secretion by ATII cells in general since it was inhibited by apyrase treatment at all stretch magnitudes examined.

Gap junction inhibitors had no effect on [^{3}H]PC secretion by ATII cells regardless of whether or not they were combined with ATI cells. We were surprised by this observation since these agents inhibited transmission of mechanically induced calcium transients from ATI to ATII cells by cultured cells (9, 35, 36). Also, Ashino et al. (3) showed using in situ whole lung microscopy that hyperinflation initiated a transient increase in type I cell cytosolic calcium that was transmitted to type II cells through gap junctions and was required to initiate lamellar body fusion with the plasma membrane. In that study, hyperinflation did not directly stretch type II cells, which underscores the physiological relevance of type I cell mechanical stimulation as a pathway to stimulate surfactant secretion. We found that ATII cells were subjected to direct mechanical stimulation in our system. Direct stimulation of ATII cells stimulates lipid insertion into the plasma membrane (25, 57) and surfactant release (59). Cell stretch also causes the direct influx of extracellular calcium, through either plasma membrane disruption or activation of channels (26, 46). Thus, in our coculture system, recruitment of calcium or inositol 1,4,5-trisphosphate from ATI cells via gap junctions was not likely to be required to initiate a calcium signaling pathway in ATII cells since this pathway would be directly stimulated by ATII cell stretch.

We found that ATI cells released ATP in response to stretch at a concentration sufficient to induce surfactant lipid secretion by ATII cells. However, Isakson et al. (35, 36) found that mechanical stimulation of an individual ATI cell with a glass micropipette did not induce a detectable level of extracellular ATP release. Thus, on a per cell basis, ATP release by mechanically stimulated ATI cells is likely to be low. However, when we stimulated the entire monolayer, the net release of ATP was significant (Fig. 6). Although the net level of ATP released into the medium is apparently low ($\sim$10–50 nM), ATP concentrations near the cell surface are typically orders of magnitude higher than bulk measurements of ATP released into the medium (6). It also has been suggested that ATP release sites and receptors may be compartmentalized into membrane microdomains to enable high local ATP concentrations (38). These two factors are likely to bring the local extracellular ATP concentration into the micromolar range required to stimulate surfactant lipid secretion (30).

In contrast to ATI cells, Isakson et al. (35, 36) found that micropipette stimulation of ATII cells induced extracellular ATP release. Because we also observed ATP release by ATII cells at high levels of stretch, this suggests that micropipette stimulation of small, cuboidal ATII cells may create focal distortions equivalent to significant changes in cell surface area. However, the same focal membrane deformation of large, flat ATI cells results in a smaller change in surface area, suggesting a rationale for the ability of the micropipette method to be more effective at stimulating ATII cells compared with ATI cells.

In summary, we found that ATI cells release extracellular ATP at a lower threshold of stretch than ATII cells and that this can serve as a paracrine pathway to stimulate surfactant secretion. Because there are many other cell types in the lung, such as fibroblasts, it is certainly possible that other cells could be sources of extracellular ATP. However, A549 lung epithelial cells release 12-fold more ATP in response to mechanical stimulation than NIH/3T3 fibroblasts (13), suggesting that the epithelium in the lung is likely the major source of extracellular ATP released in response to stretch. For alveolar epithelial cells cultured on a deformable silastic substratum, 21% stretch corresponds to 60–80% total lung capacity (54). This level of

mechanical distension is in the physiological range of a deep breath, yawn, or sigh (21, 42). Also, in the injured lung, there are likely to be hot spots where alveoli are inflated to a comparable level to compensate for atelectasis of neighboring alveoli (50). Although type I cells are more likely to be subjected to greater relative changes in cell size than type II cells in the intact lung (3), type I cell stretch might not be required in vivo since alveolar unfolding might also stimulate type I cells to secrete ATP by subjecting the cells to shear forces. Regardless of the mechanism, our results are consistent with a role for type I cells as mechanosensors in the lung and underscore the notion that type II and type I alveolar epithelial cells act in concert to regulate pulmonary surfactant secretion.

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