Interfacial sensing by alveolar type II cells: a new concept in lung physiology?

Andrea Ravasio,1 Nina Hobi,1 Cristina Bertocchi,1 Alexander Jesacher,2 Paul Dietl,3 and Thomas Haller1

1Department of Physiology and Medical Physics, Division of Physiology and 2Division of Biomedical Physics, Innsbruck Medical University, Innsbruck, Austria; and 3Institute of General Physiology, University of Ulm, Ulm, Germany

Submitted 15 October 2010; accepted in final form 23 January 2011

Ravasio A, Hobi N, Bertocchi C, Jesacher A, Dietl P, Haller T. Interfacial sensing by alveolar type II cells: a new concept in lung physiology? Am J Physiol Cell Physiol 300: C1456–C1465, 2011. First published January 26, 2011; doi:10.1152/ajpcell.00427.2010.—Alveolar type II (AT II) cells are in close contact with an air-liquid interface (IAL). This contact may be of considerable physiological relevance; however, no data exist to provide a satisfying description of this specific microenvironment. This is mainly due to the experimental difficulty to manipulate and analyze cell-air contacts in a specific way. Therefore, we designed assays to quantify cell viability, Ca2+ changes, and exocytosis in the course of interface contact and miniaturized IAL devices for direct, subcellular, and real-time analyses of cell-interface interactions by fluorescence microscopy or interferometry. The studies demonstrated that the sole presence of an IAL is not sensed by the cells. However, when AT II cells are forced into closer contact with it, they respond promptly with sustained Ca2+ signals and surfactant exocytosis before the occurrence of irreversible cell damage. This points to a paradoxical situation: a potential threat and potent stimulus for the cells. Furthermore, we found that the signalling mechanism underlying sensation of an IAL can be sufficiently explained by mechanical forces. These results demonstrate that the IAL itself can play a major, although so-far neglected, role in lung physiology, particularly in the regulatory mechanisms related with surfactant homeostasis. Moreover, they also support a general new concept of mechanosensation in the lung.

mechanical stress; pneumocytes; strain; stretch; surfactant

THE ALVEOLAR EPITHELIUM IS COVERED BY A THIN AND CONTINUOUS LAYER OF WATER (5). This aqueous layer, referred to as hypophase or alveolar lining fluid (ALF), introduces a considerable physiological relevance; however, no data exist to provide a satisfying description of this specific microenvironment. This is mainly due to the experimental difficulty to manipulate and analyze cell-air contacts in a specific way. Therefore, we designed assays to quantify cell viability, Ca2+ changes, and exocytosis in the course of interface contact and miniaturized IAL devices for direct, subcellular, and real-time analyses of cell-interface interactions by fluorescence microscopy or interferometry. The studies demonstrated that the sole presence of an IAL is not sensed by the cells. However, when AT II cells are forced into closer contact with it, they respond promptly with sustained Ca2+ signals and surfactant exocytosis before the occurrence of irreversible cell damage. This points to a paradoxical situation: a potential threat and potent stimulus for the cells. Furthermore, we found that the signalling mechanism underlying sensation of an IAL can be sufficiently explained by mechanical forces. These results demonstrate that the IAL itself can play a major, although so-far neglected, role in lung physiology, particularly in the regulatory mechanisms related with surfactant homeostasis. Moreover, they also support a general new concept of mechanosensation in the lung.

MATERIALS AND METHODS

Cell isolation and culture conditions. Cell preparations were conducted in conformity with the Austrian rules for animal care and testing (a license from the Austrian Government has been granted to T. Haller). The AT II cells were isolated from male Sprague-Dawley rats according to standard protocols described elsewhere (12, 24). For the microplate experiments (Figs. 2 and 3), isolated cells were plated in sterile multiwell tissue culture plates, for the studies with the inverted interface (Fig. 4) onto Petri dishes, and for the experiments in Fig. 5 onto glass coverslips, all left for 24 h in DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 24 mM NaHCO3, and 10% FCS (Biochrom) in a humidified 5% CO2 atmosphere of 37°C. Before the inverted interface experiments, cells were detached from the dishes by mild trypsinization (0.25%; 4 min), followed by centrifugation (1,000 rpm, 7 min) and resuspension in buffer.

Cytotoxicity. Cells were seeded in 24-well plates (Greiner) and grown to confluence. They were gently washed three times with experimental solution (see below) and exposed to air for the respective times by aspiration of the fluid out of the wells, followed by readdition of 600 µl experimental solution at 25°C and a relative humidity (RH) of 50%. After 2 h incubation, supernatants were collected and stored at 4°C. Samples were analyzed for LDH release...
according to kit instructions (Roche). Each sample of 50 μl was transferred into a 96-well plate and mixed with 100 μl assay reagent. The reaction mixture was incubated for 15 min at room temperature under light protection. Absorbance was measured at λ = 492 nm with a microplate reader (GENios Plus, Tecan, Austria).

**Exocytosis.** Cells grown in 96-wells (Sarstedt) were incubated with 1 μmol/l LysoTracker Green DND-26 (LTG) for 30 min at 37°C in DMEM. This water-soluble dye specifically, dose and time dependently, accumulates in lamellar bodies (i.e., secretory vesicles of AT II cells) where it is stably trapped by protonation, but discharged into the extracellular space once the fusion pore has formed (22, 24). After the 30-min incubation, cells were rinsed three times with experimental solution (see **Solutions and reagents**) to remove unattached cells and remaining dye. The cells were then exposed to air by sucking the entire fluid out of the wells, and left, for the indicated times, at room temperature in an atmosphere of constant relative humidity (50%) controlled by an electronic hygrometer. Experimental solution of 200 μl was readded to the cells, which was removed after 1 h and centrifuged for 3 min at 2,000 rpm. Supernatants were transferred into clean 96 wells and fluorescence of released LTG measured in a microplate reader (GENios Plus) with λex = 485 nm and λem = 535 nm and multiple reads/well.

**Phospholipid release.** Cell supernatants were collected and centrifuged as above and analyzed for phospholipid (PL) content using coupled enzymatic reactions as described (18). Briefly, supernatants were added to a buffered solution containing phospholipase D (1 U/ml), choline oxidase (0.2 U/ml), horseradish peroxidase (2 U/ml), and Amplex Red (0.1 mM), and resorufin formation was recorded with the plate reader at λexc = 540 nm, λem = 595 nm, and T = 37°C. End points of the kinetic reaction were taken after 90 min.

**Ca²⁺ measurements.** Cells were preincubated with 4 μmol/l fura 2-AM for 20 min at 37°C in DMEM, washed twice and placed into a Tecan M200 microplate reader. After 5 min, measurements were started using λexc = 335/380 nm and λem = 510 ± 20 nm. After the recording of cytosolic Ca²⁺ concentrations ([Ca²⁺]) under submerged conditions, the plate was shortly moved out of the instrument, and the supernatants were gently removed.

**Inverted interface setup and experimental procedure.** The inverted interface was previously used to analyze interfacial phenomena related with surfactant adsorption and surface film formation. For the details we thus refer to the following references: 7, 23, and 44. Briefly, a fluid is kept within a 200-μm spherically and sharply edged aperture of a sapphire cone, forming an essentially flat interface with the air below (Fig. 1A). To improve control over experimental conditions, the setup was modified as follows: A glass coverslip confined a space underneath the interface in which temperature and humidity could be controlled by a convective flow of air. Air flow was generated by a peristaltic pump connected to the inlet of the chamber. It delivered gases at rates (<2 ml/min) high enough to obtain rapid gas exchange but low enough to avoid perturbation of the interface. This was verified by fluorescent beads (2 μm) embedded at the interface showing no movements despite continuous gas-superfusion (not shown). Humidified (~100% RH) and dehumidified (~0% RH) air was produced by passing ambient air through either a water reservoir or a package of silica gel. In addition, housing of the interface and the entire stage of the microscope were thermostated (Tempcontrol 37, PeCon, Germany) yielding a measured temperature of 37 ± 0.1°C in the air and fluid sides of the interface, respectively. Before all experiments, the chamber was cleaned with water and acetone in an ultrasonic cleaner, thoroughly flushed with double-distilled water and dried with a stream of sterile air. After transfer to the microscope, the chamber was filled with 1 ml buffered solution, which immediately formed a “clean” (free of PL) IαL at the aperture plane below. The aperture was aligned and focus adjusted to the interface (7) before the cells were added on top of the buffered solution. We usually applied 50 μl of a cell suspension containing 5–20 cells. Thus contact with an IαL was accomplished by mere sedimentation of the cells toward interface, circumventing any mechanical agitation. Cytostaining of cells (Fig. 4) was obtained by overnight incubation with DiOC (2 μM) and 10 min incubation with fura 2 (4 μM).

**Dynamic interface.** A cylindrical rod (Ø 4 mm) of stainless steel was mounted on a micromanipulator (Narishige, Japan) and positioned ~100 μm above the cells grown at low density on glass coverslips. By suction, buffer solution was removed from the cells except from those right underneath the rod, so to form a central remaining drop of fluid as depicted in Fig. 1C. By modulating the height and/or lateral position of the rod, the IαL could be precisely moved while the cell(s) remained in focus. Interference resulting from light (550 nm) reflected by the interface and the upper surface of the glass (= reference beam) was used to calculate the actual thickness and steepness (slope) of the water front (see **Interferometry**).

**Microscopy.** Details of the microscopic setup including the episcopic illumination are described (7, 44). Briefly, we used a Zeiss inverted microscope (Zeiss) equipped with a monochromator (Polychrom II, TILL Photonics) and a cooled CCD camera (Imago-SVGA; TILL Photonics), both controlled by TILLVision software. Dry objectives with long-working distances were used: A ×20 Plan-Neofluar numerical aperture (NA) 0.5 to image the aperture completely, a ×40 LD-Achromplan NA 0.6 for higher resolution imaging, and a Fluor ×20 NA 1.3 for the dynamic interface experiments (all objectives from Zeiss). For fluorescence, appropriate combinations of excitation wavelengths and filter sets were used. Fluorescence images are displayed in false colors.

**Interferometry.** Interferometry was used to measure the topology (Fig. 4) and orientation (Fig. 5) of the IαL. Optical path lengths were derived from interferograms that resulted from the superposition of light being reflected by the IαL and a static reference plane. For the experiments involving static interfaces, such a reference plane was naturally provided by a reflective surface inside the objective (Fig. 5).
The static situation allowed the application of phase stepping, where the phase of the reference beam was repeatedly stepped by a known increment (25). This results in a set of fringe patterns that can be mathematically combined to extract the optical path length. For each situation, three interferograms were recorded with reference phase values of 0, 2π/3, and 4π/3 radians. The phase stepping was performed by controlled axial movements of the objective lens in the submicrometer range. To ensure a high accuracy of the objective movements, a special apparatus involving a level and a manual micrometer stage was attached to the microscope’s focus knob. A He-Ne laser (633 nm) was used for these experiments.

The dynamic interface required an alternative method for fringe pattern evaluation. There, the thickness of the water layer was changed quickly by movements of the steel rod (Fig. 1C); hence, there was not enough time to perform phase stepping. The high density of fringes and the relatively smooth phase topography of the water layer, however, allowed determining the layer thickness from a single interferogram using Hilbert phase demodulation (28). From an obtained phase map, the mean slope of the IAL was extracted. This parameter was used as an estimate for the thickness of the water layer that separates the cell from the surrounding air; i.e., the smaller the slope, the thinner is the remaining aqueous layer, and the higher the force exerted on the cell. Absolute slope was normalized between 1 (fringes not detectable: thickness of water layer exceeding the coherence length of the interfering light) and 0 (distance between single fringes approaching ∞: interface parallel to the glass coverslip).

Normalization was necessary to eliminate differences in the lateral extension of the water drop (contact angle) together with differences in the heights of the cells and was done by developing own Matlab algorithms. A reduction in the normalized slope thus denotes a progressive steepening of the water front, no change denotes an interface that cannot be flattened further because of the resistance exerted by the cell(s) underneath. These measurements were combined with ratiometric fura 2 measurements as described above.

Solutions and reagents. The standard experimental solution contained (in mM) 140 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 5 glucose, and 10 HEPES (pH 7.4 at 25°C). The Amplex Red Phospholipase D assay kit, BAPTA-AM, BCECF-AM, 2,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl-1-hexadecanoyl-sn-glycero-3-phosphocholine (BODIPY-PC), 3,3’-dioctadecyloxycarbocyanine perchlorate (DiOC6), calcine-AM, FM 1–43, fura 2-AM, Lyso-Tracker Green DND-26, and phorbol 12-myristate 13-acetate (PMA) were purchased from Invitrogen Molecular Probes (Austria); ATP, gadolinium, thapsigargin, EGTA, DMEM, IgG, phospholipase D, Triton X-100, and trypsin were from Sigma-Aldrich; FCS was from Biochrome; the LDH detection kit was from Roche; elastase (for the measurement of free water surface and/or an increase in barrier/resistance for evaporation) was obtained (in mM) 140 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 5 glucose, and 10 HEPES (pH 7.4 at 25°C). The Amplex Red Phospholipase D assay kit, BAPTA-AM, BCECF-AM, 2,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl-1-hexadecanoyl-sn-glycero-3-phosphocholine (BODIPY-PC), 3,3’-dioctadecyloxycarbocyanine perchlorate (DiOC6), calcine-AM, FM 1–43, fura 2-AM, Lyso-Tracker Green DND-26, and phorbol 12-myristate 13-acetate (PMA) were purchased from Invitrogen Molecular Probes (Austria); ATP, gadolinium, thapsigargin, EGTA, DMEM, IgG, phospholipase D, Triton X-100, and trypsin were from Sigma-Aldrich; FCS was from Biochrome; the LDH detection kit was from Roche; elastase (for the AT II cell preparation) was from Elastin Products; and PFD (perfluoro-10 HEPES (pH 7.4). The Amplex Red Phospholipase D assay kit, BAPTA-AM, BCECF-AM, 2,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl-1-hexadecanoyl-sn-glycero-3-phosphocholine (BODIPY-PC), 3,3’-dioctadecyloxycarbocyanine perchlorate (DiOC6), calcine-AM, FM 1–43, fura 2-AM, Lyso-Tracker Green DND-26, and phorbol 12-myristate 13-acetate (PMA) were purchased from Invitrogen Molecular Probes (Austria); ATP, gadolinium, thapsigargin, EGTA, DMEM, IgG, phospholipase D, Triton X-100, and trypsin were from Sigma-Aldrich; FCS was from Biochrome; the LDH detection kit was from Roche; elastase (for the AT II cell preparation) was from Elastin Products; and PFD (perfluoro-

**RESULTS**

**Exposure to air is deleterious.** AT II cells, grown to confluence on a glass coverslip, were preincubated for 30 min with calcine-AM, and the coverslip was placed for 20 min in vertical position into buffered solution so that half of it was exposed to air. Afterwards, cells were rinsed and stained with FM 1–43. The result (Fig. 2A) demonstrated a loss in calcine (green) and a gain in FM 1–43 (red) in the air-exposed region. Since calcine is retained in intact cells and FM 1–43 enters damaged cells, the combination of both is an indicator of plasma membrane integrity and compellingly demonstrates the deleterious effect of air contact. However, this simple approach did not allow a reconstruction of the position of the IAL nor a quantitative description of its effects, and thus has not been pursued further. Instead, we performed microplate experiments to analyze cell damage more quantitatively and on a time-resolved basis. LDH release in AT II cells was not measurable before 20 min air exposure (Fig. 2B). Notably, at this time, cells were already partially exsiccated (see Fig. 2C). Longer exposure times (40 min) increased LDH up to amounts comparable to those released by 1% Triton X-100 (equals 100% cytotoxicity). This proved, on a time-resolved basis, a similar extent of cell damage as shown before (Fig. 2A). Taking these findings together, we conclude that AT II tolerate air contact for more than 10 min after which cell membrane integrity is lost.

**Exposure to a stimulus.** First indication of increased exocytosis (i.e., release of LTG through exocytotic fusion pores, see MATERIALS AND METHODS) was at 30 s (Fig. 3A). This suggests that air contact stimulates the cells and is effective at a time when cell damage can be ruled out. It also suggests that cells respond before they may start to actually “dry out” (Fig. 2C). The profound increase in LTG release after cell damage (20 and 40 min) can be explained by a loss of plasma membrane integrity together with that of the limiting LB membrane.

**Fig. 2. Air contact is deleterious.** A: AT II cells on a glass coverslip partially submerged in buffer. Costaining with calcine (green) and FM 1–43 (orange) demonstrates cell damage in the air-exposed region (top; 20 min exposure). B: time dependence of cytotoxicity (100% = LDH-release in 1% Triton) measured in microplates. Significant (P < 0.01) levels compared with controls (Co; without air contact) are marked as black bars (n = 4, means ± SD). Dashed line indicates the approximated transition from interface contact (10 s-5 min) to exsiccation (>5 min air exposure) as described in C. C: weight loss (evaporation) of wells containing buffer or a confluent cell monolayer from which fluid was entirely removed by aspiration. Evaporation of buffer was measured but constantly decreased on a cell monolayer. At 10 min, this difference was already significant (P < 0.05; n = 4), indicating onset in partial reduction of free water surface and/or an increase in barrier/resistance for evaporation (e.g., by the cell membrane), thus denoting incipient exsiccation.
with subsequent discharge of LTG into the cell exterior. This is also supported by the signals that considerably overrun those produced by ATP + PMA, two potent secretagogues that usually yield a high exocytotic response (53).

Similar with the time course of exocytosis was the release of PL (Fig. 3B). The first measurable increase was at 30 s, the highest (within the period of cell survival) at 10 min, and all values are in the range of response elicited by ATP + PMA. However, the time course of PL release appeared biphasic, with a return to control values at 2 and 5 min (the same tendency may be seen in exocytosis). A further difference is the massive LTG release at times 20 and 40 min, which is not reflected by a similarly pronounced increase in the amount of released PL. A likely explanation is the kind of membrane damage: it may be extensive enough to discharge the low-molecular-weight compound LTG out of lamellar bodies (and LDH out of the cytosol) but not extensive enough to release the bulky and essentially water-insoluble surfactant aggregates (∼2 μm) into the extracellular space.

The stimulus is Ca2+ dependent. The exocytosis assay was repeated with BAPTA-loaded cells (Fig. 3C). The result revealed that in the absence of an air stimulus, constitutive secretion was identical in control and BAPTA-treated cells. After 1 min air exposure, however, BAPTA-treated cells exhibited a significantly reduced secretory response, suggesting a regulated exocytosis induced by contact with air. However, BAPTA was unable to block exocytosis completely and was more effective in ATP-stimulated cells. Both results suggest a significant contribution of an additional, Ca2+-independent path. Astonishingly, BAPTA also blocked exocytosis at time 40 min, when cell damage was considerable (Fig. 2B). The obvious Ca2+ dependence was further analyzed by continuous measurement of the [Ca2+]c using fura 2-loaded cells (Fig. 3D). All measurements showed a small but significant [Ca2+]c elevation immediately after suction of the fluid, probably responsible for the early initiation of exocytosis. A second, more sustained and pronounced [Ca2+]c elevation occurred after ∼5 min, reaching values exceeding those of the Ca2+-agonist ATP (not shown). The source of the bulk Ca2+ change was clearly extracellular: Washout of Ca2+ (Ca2+-free buffer) before air exposure abolished the rise in the fura 2 ratio, similar to the effect of gadolinium in Ca2+-containing buffer. Furthermore, thapsigargin (tg), which depletes intracellular Ca2+ stores, had no inhibitory effect. Perfluorodecalin (PFD) was used in these experiments as a model for a liquid-liquid interface. It had no effect except the initial rise in [Ca2+]c.

Microscopy of cell-interface interactions. So far, the microplate experiments revealed a highly significant, time- and Ca2+-dependent exocytotic response of AT II cells to air contact followed by massive cell damage. But what are the mechanisms, and what are the signals? To approach these questions, we used a static, inverted IAL (Fig. 1A) for the following reasons: 1) Cells, applied on top, sediment toward that interface and contact it with the least possible mechanical force (7). 2) Motions of cells abruptly stop when they reach the focal plane, allowing to precisely determine the instance of contact. 3) Cells can be imaged by brightfield, reflection, epifluorescence, and interference without focal shifts or loss of cells out of view. 4) Temperature (37°C), humidity (∼100 or ∼0%), and gas composition (ambient or N2) in the air compartment could be adjusted. 5) Finally, and importantly, problems associated with osmotic changes can be ruled out because of a constant and large (1 ml) fluid volume in the chamber.

The results from the static interface experiments can be grouped into 3 classes: The first is no response (Fig. 4A) and this is when a cell gets close to an IAL, at 100% rH (scheme). The cell neither deforms nor penetrates it (interferometry), the intracellular Ca2+ does not change, there is no dye leakage out of the cells (fura 2 ratio), and exocytosis is not measurable (% DiOC). However, it may still proceed at a slow pace, as it leads to detectable amounts of PL surrounding the still intact cells.

**Fig. 3.** Air contact is a Ca2+-dependent stimulus. A: Exocytosis, measured as LysoTracker Green DND-26 (LTG) release from cells in microplates, was significant after 30 s air contact (controls and ATP/PMA-stimulated cells were without air exposure; n = 4). B: release of surfactant phospholipid (PL), same conditions as in A (n = 5). C: inhibition of exocytosis (as shown in A) by BAPTA. Inhibition was less pronounced than in ATP-stimulated cells without air exposure (n = 5, means ± SD). D: source of Ca2+ signals. Cells were preincubated under the indicated conditions: Ca2+ (standard experimental solution, n = 9), Ca2+-free (0 Ca2+, 1 mM EGTA, 5 min, n = 7), thapsigargin (tg, 100 nM, 5 min, n = 5), tg Ca2+-free (100 nM thapsigargin in 0 Ca2+, 1 mM EGTA, 5 min, n = 7), gadolinium (50 μM, 20 min, n = 7), and PFD (perfluorodecalin), added at time 0 (n = 9). PFD served as a control for a liquid-liquid interface. Levels of significance in A–C; no significance (white bars), P < 0.05 (gray bars), P < 0.01 (black bars).
after 24 h (Fig. 4D). The lack of an immediate Ca\(^{2+}\) signal suggests that interface contact per se is not sensed by the cells, and precludes nanometer-scaled electrochemical or osmotic gradients [which are described in thermodynamic models of the interface (32)], surface-associated gradients of respiratory gases (tested by perfusion with N\(_2\), not shown), or the surface tension itself as reasons for an interfacial sensing. These experiments also demonstrate that immediate (0–10 min) and intimate (nm-m) interface contact is neither a threat nor a stimulus, even when surface tension \(\gamma\) of the interface is unphysiologically high (\(\sim 70\) mN/m).

The second is response (Fig. 4B and supplemental video S1 shown online at the AJP-Cell Physiol website). When the distance to the IAL is further reduced by applying 0% rH, the
intracellular Ca\(^{2+}\) rose quickly, and secretion was enhanced in most but not all cells. Despite the sustained [Ca\(^{2+}\)]\(_c\), which did not fully revert to precontact levels, there were no signs of cell damage. We assume that evaporative water loss forces cell structures, such as the glycocalyx and other macromolecules that extend into the extracellular space, into ultimate close distances to the interface, probably beyond a remaining hydration shell (20). This configuration is highlighted by the distortion of interference fringes at the site of a cell. When analyzed, this amounted to actual surface deformations in the range of several (~70) nm. Thus, in configuration B, the cell surface is in obvious contact with the interface and probably subject to forces resulting from these interfacial deformations. The positive effect of 0% rH was additionally confirmed by changing rH (100% to 0%) during continued perfusion of the air compartment (practically, cells in condition A were subject to condition B; not shown). Theoretically, changing to 0% humidity could also have influenced local T at the utmost surface zone of the IAL. However, the experiments were performed in thermal equilibrium with a high heat transfer to the chamber (thermostat plus air convection), and AT II cells did not respond to a sudden cooling down when tested independently, thus essentially ruling out that [Ca\(^{2+}\)]\(_c\) signals were evoked by a local change in T.

The third is rupture (Fig. 4C and supplemental video S2). In seldom scenarios (4–5 cells) and never at 100% rH, we have seen an actual penetration of the interface followed by cell rupture and loss of cytosolic dye. We therefore conclude that penetration of the interface exposes the entire cell, or part of it, to the surface tension that ultimately leads to irreversible cell rupture. The main force acting here (besides gravitation and buoyancy as in Fig. 4, A and B), is surface tension with the lateral component of it being sufficient to tear a cell apart (cell rupture, however, was often seen with other cells, e.g., erythrocytes and monocytes, not shown).

So far, we have shown that intimate interface contact per se (Fig. 4A) is not the stimulus. On the other hand, surface penetration (Fig. 4C) is a deleterious event.

**Dynamic interface.** The hypothesis remains that the sensory event is associated with a bending or a corrugation of the interface (provoked, e.g., by situation in Fig. 4B), exerting a mechanical load. To test this assumption further, we applied a dynamic interface model. With this setup, the IAL, visualized by interferometry, could be precisely moved with respect to the cells while monitoring [Ca\(^{2+}\)]\(_c\) (Figs. 1C, 5, 6, and supplemental video S3). The results principally confirmed those of the static measurements: AT II cells do not respond up to a certain distance to the interface (white symbols in Fig. 5, A and B).

---

**Fig. 5. Dynamic interface experiments.**

A: provoked interface contact and ensuing Ca\(^{2+}\) changes (see also supplemental video S3.mov). Slope of the interface (symbols) was calculated from the interference fringes shown in D (see also Fig. 1C and text). The lower the value, the flatter and closer is the waterfront above the cells and, consequently, the higher the force acting on the cells. White symbols denote the advancement of the interface to the cells, gray symbols the bending of the interface above the cells, and black symbols its retraction from the cells. [Ca\(^{2+}\)]\(_c\) is indicated by black lines. It increased during interface contact (plateau region, gray symbols: interface was maximally close to the cells although the rod was continuously moved farther away from the cells). [Ca\(^{2+}\)]\(_c\) recovery did not start until the interface was retracted (black symbols). Complete cell rupture (left) was only seldomly observed. B: [Ca\(^{2+}\)]\(_c\) as a function of the normalized slope of the interface (data from A). C: waterfront, once in intimate contact with the cells, was repetitively moved forth and back, creating Ca\(^{2+}\) oscillations (black lines) in the 3 cells shown in D. D: brightfield/interference (top) and fura 2 ratio images (bottom) of AT II cells during advancement of the IAL, (left) and during oscillatory movements (right).
Onset of \([\text{Ca}^{2+}]_c\) increase (lines in Fig. 5A) was observed, however, when the interface started to bend over the cells (gray symbols). Moving the rod farther from the cells lead to \([\text{Ca}^{2+}]_c\) increase of different magnitudes or even to cell rupture in rare cases \((n = 2)\), demonstrating that the bended interface exerts a force. Moving back the waterfront toward the cells (black symbols) resulted in \([\text{Ca}^{2+}]_c\) recovery (except after cell rupture). Finally, and most intriguingly, when the IAL, kept at a minimum distance to the cells, was repetitively moved back and forth in small increments, the cells responded with out-of-phase \([\text{Ca}^{2+}]_c\), oscillations (Fig. 5, C and D; and supplemental video S3).

**DISCUSSION**

Quantitative microplate experiments showed that exposure of AT II cells to air provokes a significant, time- and \(\text{Ca}^{2+}\) dependent exocytotic response. Specialized microscopic investigations then revealed that the mechanism of stimulation is not based on interface contact per se, which did not activate the cells, ruling out that surface-associated gradients may play a role. However, the effect can be sufficiently explained by mechanical stimulation and signal transduction initiated whenever surface forces act on subcellular structures. We thus propose a model of mechanosensation in AT II cells that integrates the IAL as an essential part of it. The model and its role. However, the effect can be sufficiently explained by mechanical stimulation and signal transduction initiated whenever surface forces act on subcellular structures. We thus propose a model of mechanosensation in AT II cells that integrates the IAL as an essential part of it. The model and its implications in lung biomechanics are sketched in Fig. 6 and critically discussed below. Before, we want to list some facts and evidence in favor of this new concept.

1) Undoubtedly, the IAL is an integral part of the lungs. With some exceptions (26), consensus exists that its extension is continuous, being flat over flat alveolar regions and bended at its corners, demonstrated by low-temperature electron microscopy (5) and optical-sectioning microscopy (34). Numerous ultrastructural and microscopic analyses also tell that AT II cells are cuboideal and preferably located near the septal corners with their apical side extending luminal (41, 52). A few investigations exist suggesting that AT II cells, including their microvilli, are entirely submerged by the ALF, which is even bulged into the alveolar lumen at the cell apex (5, 20). Taking these facts and evidence together, a picture of an interfacial environment, sketched in Fig. 6A, emerges. From this model, one must conclude that AT II cells are constitutively close to the interface and that the interface is bended at the site of these cells. A bended interface above AT II cells would even correspond with the “dry lung” model put forward by Hills (26). In fact, for the “normal” AT II cell’s microenvironment, essentially no other model has been proposed than that depicted here.

2) Surface tension in the lung is dramatically reduced by pulmonary surfactant, but not completely abolished, and may even reach \(~30\) mN/m at total lung capacity \([\text{TLC}]; (45)\). According to the laws of Young and Laplace, a water surface of defined surface tension and defined curvature results in a net force acting perpendicular to it. Assuming a concave interface with a radius of curvature of \(20\) μm, similar to that depicted in Fig. 6A, and a surface tension of \(30\) mN/m, this net force would amount to \(3\) kPa of a transmural pressure. This value is far from being negligible but instead in the range of the recently reported elastic modulus for the nuclear \((3.1\) kPa) and cytosolic \((4.7\) kPa) part of an AT II cell as measured by AFM elastography (3). In further consideration that this elastic modulus showed a multimodal distribution over the cells \((\text{from} \sim 1\) to \(\sim 14\) kPa), it means that the pressure gradient of a bended interface would be sufficient to exert a considerable deformation, compression, or any other mechanical burden to at least parts of the cells.

3) Considerable evidence exists that the interface, due to surface forces, is a constant modulator and determinant of alveolar geometry and microstructure, exerting a molding effect on tissue elements (4). Surface forces are so strong that the configuration of capillaries, and hence the microcirculation, is changed. In severe pathophysiologic situations, high surface tensions even lead to alveolar collapse. Moreover, an investigation on pulmonary macrophages convincingly demonstrates that at high surface tensions, these mobile cells are virtually squeezed into the alveolar corners, turning them immobile and inactive (1). This again demonstrates the magnitude of force acting at this microscopic scale and its effects on the cells.

4) It is known that lung inflation leads to an increase in surface tension, elegantly shown by Schürch et al. (45) in intact lungs. The best explanation is an increase in surface area of the respiratory IAL, probably in consequence of tissue stretch. Uncertainties existed only with regard to the percentage of TLC at which unfolding of membrane pleats pass into effective tissue stretch (49), and whether this might be a continuous or a threshold function of TLC (2, 15). The discovery of alveolar recruitments/derecruitments during a respiratory cycle then added an additional level of complexity (37). Recently, Perlman and Bhattacharya (41) found that the alveolar expansion pattern is markedly nonuniform between the tissue elements, even at the level of one single alveolar unit. Thus, although consensus now exists that lung inflation leads to alveolar expansion and tissue stretch, the details are still not precisely known (19, 48).

![Fig. 6. Concluding summary. Schematic representation of an alveolar corner (A and B) and its simulation by our in vitro approach (C and D).](http://ajpcell.physiology.org/)
Finally, it is experimentally documented that AT II cells are mechanosensitive and respond to cell stretch with Ca\(^{2+}\) increase and surfactant secretion (15, 54). An unresolved dispute only exists whether tissue stretch is sensed by the AT II cells directly or indirectly [via type I cells; (2)]. As proposed and demonstrated experimentally (2, 41), the geometry of the alveolar predisposes the type I cells as the sensor and the type II as the effector cells in tissue stretch.

Taking into account all facts and evidence listed in 1–5, and the experimental results presented here, we suggest that AT II cells due to their specific cell morphology, location, and function are the preferred “site” to sense and interact with the respiratory $I_{\text{AL}}$. The hypothetical model we propose includes the following: lung inflation leads to a thinning of the ALF and a concomitant increase in surface tension. Either thinning or surface tension, but most likely both in concert, impose a pressure onto the apical side of AT II cells that contains or is connected with mechanosensitive elements. Activation of these elements initiates the intracellular pathways leading to enhanced secretion of surfactant. Released surfactant replenishes the subsurface-associated surfactant pool, enforces the adsorption of new surfactant onto the interface, and diminishes its surface tension, thereby reducing the initial strength of the stimulus (theoretically, surfactant may have the additional function to stabilize and retain a certain amount of fluid in the alveolar corners, but this is unproven). In this model, lung inflation would not be exclusively sensed as tissue stretch by the alveolar type I cells, but additionally, or even primarily, by direct interaction of the interface with the AT II cells. An allowedly indirect, but intriguing support of this model, comes from the observations that macrophages and capillaries are compressed and flattened into the epithelial surfaces at high surface tension (1). The model would also consolidate the two conflicting hypotheses about mechanosensing as described above. And finally, since interfacial sensing is related to the amount of fluid, at least in the alveolar corners, this model would also propose a hypothetical mechanism by which the actual status of the ALF volume could be continuously monitored by a defined biological entity. Such a sensing system including a mechanistic way of interaction of a physical phase boundary with a defined cell has not been described before.

It would be interesting to compare the magnitudes of an apically applied interfacial stress with that attributable to basolateral deformations due to basement membrane stretch. If we take the reported mean cytoplasmic elastic modulus of AT II cells of 4.7 kPa (3), and if we further assume that a 40% rise in alveolar epithelial surface area (49) would be transduced to the same extent into basolateral extension of an AT II cell (~6% increase in one dimension), application of the Young’s modulus would yield a pressure of 0.3 kPa acting on the cells. Compared with the 3 kPa exerted by our exemplary, bended $I_{\text{AL}}$ of 20 μm radius and 30 mN/m (as calculated above), one would have to conclude that surface forces are about one order of magnitude above the forces exerted by tissue stretch, even at 100% TLC. However, this is a gross estimation containing many uncertainties and not taking into account, e.g., heterogeneities in alveolar deformation, actual values of surface tension, differences in stiffness properties between the microvillar region and its basolateral counterpart, or the actual contour of the ALF. Alternatively, the pressure exerted by the interface would compare well with the reported maximum transpulmonary pressure of 3 kPa at TLC. But also this comparison does not take into account that transpulmonary pressure is not the pressure acting on AT II cells solely but also depends on several additional factors like surface tension or the elastic modulus of the connective and other tissues. So far, we can only speculate that surface forces are in a comparable range than those exerted by tissue stretch.

However, we are aware that this model contains some assumptions, related to the experimental inaccessibility of the alveolar structure including the epithelium, the ALF, the surface coat, and the air. First, lung inflation may not lead to a thinning of the ALF. With regard to this point, no report provides direct data. The authors even think that proof of this assumption cannot be done experimentally: It would necessitate that changes of the thickness of the ALF in alveolar corners in the range of a few hundred nanometers be measured with high precision during the respiratory cycle, which is a highly difficult task. However, the above objection can be excluded by theoretical considerations: If the thickness of the ALF would be rather constant despite surface changes, the alveolar fluid would have to be absorbed during exhalation and replenished during inspiration, a mechanism that lacks any physiological ground. In line with this, Bastacky et al. (5) and Lindert et al. (34) arrived to a similar conclusion when they argued that ALF thinning has to be expected by increasing the lung volume toward TLC. Second, in the lung, surfactant concentration is so high and surface tension is so low that interface contact might not be sensed at all. This objection cannot be ruled out completely. Further investigations along this are in progress; however, they require substantial technical improvements that allow modulating surface tension, even down to the minimum values observed in maximally compressed monolayers, while modulating the distance of the cells to the interface and monitoring interference in parallel. This task could not yet be solved in a sufficient way: Any addition of a surfactant (like Curosurf) led, due to strong light refraction effects, to a concentration-dependent blurring and finally loss of interference signals. Thus Curosurf could only be used at such low concentrations (0.1 mg/ml) where its effect on reduction of surface tension was probably too small to eliminate a deforming stress (6). Third, our model does not take into account intercellular effects due to mechanical deformation: Ca\(^{2+}\) propagation from type I (2) or endothelial cells (51), as well as paracrine stimulation of AT II cells via ATP (39), are important mechanisms in the general stress response. However, our results suggest that mechanical deformations may become manifest on different levels of tissue organization, including the $I_{\text{AL}}$, as an important part of it, and the picture on intra-alveolar signalling may be not complete unless all potential components are included.

One has also to keep in mind that the ALF is not a static layer, but, according to recent findings, subject to a continuous convective flow even when the lungs were held at constant inflation pressure (34). This convective flow may be further subject to periodic oscillations imposed by the breathing cycle or by the transit of erythrocytes. From our experiments, it appears that the interface elicits a cell response when the resulting net force acts perpendicular to it, which means, when the interface leads to a compression of the cells. However, we cannot exclude that this stimulus also includes a lateral component, leading to a deflection of cellular structures rather than...
their compression, which would correspond to shear stress. In analogy to the inner hair cells of the cochlea, the microvilli of AT II cells on the cell apex would be perfect candidates to sense those lateral components, but this is a farfetched speculation.

A specific and novel result of our study is the increase of \([Ca^{2+}]_c\) upon interface contact, which has been demonstrated by three approaches independently (microplate measurements, inverted and dynamic interface experiments). We used those \(Ca^{2+}\) measurements because changes in \([Ca^{2+}]_c\) have already been demonstrated to be essential in alveolar mechanosensing (2, 15, 54), and because \([Ca^{2+}]_c\) is the key messenger in regulated exocytosis (9). In previous investigations, it has been shown that \([Ca^{2+}]_c\) increase is due to \(Ca^{2+}\) influx, probably stretch-activated \(Ca^{2+}\) channels, whereby store-operated pathways act in concert (15). Gadolinium, a nonspecific blocker of mechanosensitive calcium channels, abolished this \(Ca^{2+}\) activation in our experiments, but also strain-induced fetal rat lung cell proliferation (36), suggesting activation of a common cation channel in strain- or interface challenged cells (50). Further pharmacological profiling of this signalling mechanism in combination with silencing strategies would be needed to elaborate the similarities/dissimilarities between tissue stretch and interfacial sensing. In contrast to stretch activation (15), however, \([Ca^{2+}]_c\) increase in our experiments seems to be sustained. On one hand, sustained signals correlate with sustained surfactant secretion (10, 16, 22) or may even be a necessary determinant for normal lung growth and development (8), they also might be apoptotic signals or might lead to proliferation (35) or the initiation of inflammatory processes (43), beside many others (48). At the moment, we have no explanation for the reasons and consequences of these sustained signals (except secretion), which have to be investigated by comprehensive studies further.

\(I_{\text{AL}}\) cultures are abundantly used for various cell types that may have constitutive or acute contact with air; e.g., bronchial and tracheal airway epithelial cells (33), nasal mucosal cells (11), middle ear epithelial cells (42), gastric surface mucus cells (38), various cell lines representing any part of the respiratory tract (21), lung slices (46), but also skin fibroblasts and epidermal keratinocytes (47), to give some examples. The techniques used range from simple hydrogels to filter-based systems up to highly specialized devices (27) and are applied for long-term studies in most cases. However, despite the variety of cells and methods used, there is a common finding of a mostly clear-cut effect of an \(I_{\text{AL}}\) on specific [e.g., channel activity (30)] or global [e.g., differentiation (14)] cell functions. From these results and the physiological importance of such common cell environments, it would be desirable to obtain further mechanistic insights into the underlying bio-physical signalling events.

In conclusion, we introduce a biological sensing system that describes, for the first time, the effect of an \(I_{\text{AL}}\) on a physiologically pivotal effector cell. Further studies will be directed toward the elucidation of physical and molecular mechanisms involved therein, and the role of surfactant in this system. Other studies will show whether this model has implications in addition to the one described here; e.g., in the regulation of alveolar fluid balance or the induction of local fibroptic processes, or whether it can be extended for other types of cells that may experience air contact in a similar way than the type II cells of the lungs.

ACKNOWLEDGMENTS

Technical assistance by Gerlinde Sibler and Irina大纲 is gratefully acknowledged.

Present address of A. Ravasio: Division of Genomics and Genetics; School of Biological Sciences, Nanyang Technological University, Singapore; Present address of C. Bertocchi: Mechanobiology Institute Singapore, National University of Singapore, Singapore.

GRANTS

This work was supported by the Austrian Fonds zur Förderung der Wissenschaftlichen Forschung, projects P17501 and P20472.

DISCLOSURES

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

REFERENCES


highly selective sodium channels in alveolar type II cells is determined by port across adult alveolar epithelial cells: Effects on Cl


receptor and transport function in cultures with an apical air interface.


Sensing an air-liquid interface.

Sensing an air-liquid interface.