The presence of intercellular Ca\(^{2+}\) type 1 cells by gap junctional (1, 13) or paracrine (10, 12) may also be communicated to the type 2 cell from adjoining alveoli. However, it is not known whether the acinus coordinates surfactant secretion by regulating signal communication between adjacent alveoli.

Here, we addressed this question through the application of a photo-excited release of caged Ca\(^{2+}\) in alveoli in situ. Photo-excited, intracellular release of caged compounds provides a means for localized delivery of agonists without incurring nonspecific effects of receptor ligation (20). For Ca\(^{2+}\) uncaging, cells were loaded with membrane-permeable o-nitrophenyl EGTA-AM (NP-EGTA-AM), which deestersifies intracellularly to NP-EGTA. Because of its high Ca\(^{2+}\) affinity (K\(_{d}\): 80 nM), NP-EGTA acts as a “cage,” sequestering free Ca\(^{2+}\) (6). Photo excitation with high-intensity UV light dissociates NP-EGTA into iminodiacetic products of low Ca\(^{2+}\) affinity (K\(_{d}\): 3 mM), thereby releasing the caged Ca\(^{2+}\) and increasing [Ca\(^{2+}\)]\(_{cyt}\).

Here, through the first application of these methods in intact alveoli, we found that an increase of Ca\(^{2+}\) in one alveolus was communicated to the neighboring alveolus, where it induced LB exocytosis. Surprisingly, the stimulated exocytosis rate was independent of the magnitude of the Ca\(^{2+}\) stimulus.

**METHODS**

Fluorescent dyes and reagents. The fluorophores were fluo 4-AM, fura 2-AM, LysoTracker green (LTG), LysoTracker red (LTR), and 2,7-bis(carboxyethyl)-5(6)-carboxyfluorescein-AM (BCECF-AM) (Molecular Probes, Eugene, OR). The cell-permeable Ca\(^{2+}\) cage was NP-EGTA-AM (Molecular Probes). The vehicle for dyes was HEPES buffer (150 mmol/l Na\(^{+}\), 5 mmol/l K\(^{+}\), 1.0 mmol/l Ca\(^{2+}\), 1 mmol/l Mg\(^{2+}\), and 20 mmol/l HEPES at pH 7.4) containing 4% dextran (70 kDa), 1% FBS, and 2% reconstituted bovine surfactant. Agents were xestospongin C (Calbiochem-Novabiochem), pyridoxal phosphate-6-azobenzene-2,4-disulphonic acid tetrasodium salt (PPADS), and ATP (Sigma Chemical, St. Louis, MO).

Real-time digital imaging of lung alveoli. Animal procedures were approved by the Institutional Animal Care and Use Committee of St. Luke’s-Roosevelt Hospital Center. Using our reported methods (11, 19), we pumped perfused lungs from anesthetized Sprague-Dawley rats (3.5% halothane inhalation and 35 mg/kg ip sodium pentobarbital) with autologous blood (14 ml/min) at 37°C. Baseline pulmonary artery and left atrial and airway pressures were held constant at 10, 5, and 5 cmH\(_{2}\)O, respectively. We viewed alveoli by means of an image-intensifier (Midnight Sun, Imaging Research, St. Catharine’s, ON, Canada) mounted on a fluorescence microscope (AX-70, Olympus America, Melville, NY) and quantified alveolar fluorescence using image analysis software (MCID 6, Imaging Research). We identified alveolar margins under bright-field conditions.

For alveolar loading, we micropunctured single alveoli and micro-infused the following: 1) fluo 4-AM (5 μM) to detect cytosolic Ca\(^{2+}\) (excitation at 495 nm), 2) fura 2-AM (10 μM) to quantify [Ca\(^{2+}\)]\(_{cyt}\) by the ratiometric method (11) (alternate excitations at 340 and 380 nm),...
and 3) LTG (75 nM) to detect alveolar type 2 cell secretion (excitation at 495 nm). In some experiments, we used LTR (50 nM, excitation at 550 nm) as a type 2 cell marker (25). All dyes were loaded by a 30-min infusion with or without the caged Ca\(^{2+}\) compound NP-EGTA (100 \(\mu\)M). We obtained images at intervals of 10 or 20 s, respectively, for fluo 4 and LTR. In fura 2 experiments, the fluorescence ratio was calibrated using a fura-Ca\(^{2+}\) \(K_o\) of 224 nmol/l (11). In alveoli loaded with more than a single dye, we confirmed the absence of cross-excitation. Inhibitors were infused for 30 min together with the dye and Ca\(^{2+}\) cage.

To determine the type 2 cell phenotype and location in the intact alveolus, we followed our reported intra-alveolar microinjection protocol (1). First, we microinjected an anti-type 2 cell-recognizing mAb (50 \(\mu\)g/ml, 10 min; a gift of Dr. L. G. Dobbs, Cardiovascular Research Institute, Department of Medicine and Pediatrics, University of California, San Francisco, CA). We then injected fluorescent Alexa fluor 488-labeled goat anti-mouse IgG (Molecular Probes; 10 \(\mu\)g/ml, 4 min). After a 1-min washout of fluorescent dye with vehicle, we recorded the fluorescence of type 2 cells.

For the inhibition of connexin43 (Cx43) gap junctions, we used the Cx43-recognizing peptides gap 26 (amino acid sequence: VCYDKSFPISHVR) and gap 27 (SRPETKITTIFII) in conjunction with the Ca\(^{2+}\)-conductive peptides gap 26 (190 nM) and gap 27 (160 nM) as a mixture that we microinjected for 30 min together with the Ca\(^{2+}\) cage.

Photo-excited Ca\(^{2+}\) uncaging. For the photo uncaging of NP-EGTA, high-intensity light flashes generated by a UV lamp (JML-C2, Rapp OptoElectronic, Hamburg, Germany) were directed through the microscope objective at the NP-EGTA-loaded alveolar target. Uncaging occurred in a circle of diameter 70 \(\mu\)m, as calibrated by directing the UV beam on a micrometer slide, in 20–30 s.

Statistics. Group data are means \(\pm\) SE. Results were replicated at least three times in at least two different lungs unless otherwise stated. Differences between groups were tested by paired \(t\)-test for two groups and by the ANOVA-Newman-Keuls test for more than two groups. Significance was accepted at \(P < 0.05\).

**RESULTS**

**Ca\(^{2+}\) conduction between alveoli.** By means of alveolar micropuncture, we coloaded alveolar epithelial cells with the Ca\(^{2+}\) cage NP-EGTA and the Ca\(^{2+}\) fluorophore fluo 4. Photo excitation of the cage-loaded cells caused major increases of fluo 4 fluorescence not only in the targeted area (diameter 70 \(\mu\)m) but also in all cells of the alveolar wall (Fig. 1, A–C). To rule out nonspecific effects, we affirmed that no fluorescence increases occurred in alveoli not loaded with the Ca\(^{2+}\) cage (Fig. 1A). In the targeted region of cage-loaded alveoli, photo excitation increased [Ca\(^{2+}\)]\(_{cyt}\) by 96 \(\pm\) 10 gray levels (\(P < 0.05, n = 5\)), which then decayed to baseline with a half-time of 3.3 \(\pm\) 0.3 min (Fig. 1, B and C). A second photo excitation at the same site failed to increase fluorescence, indicating that a single stimulus completely uncaged the targeted region (Fig. 1B).

However, uncaged Ca\(^{2+}\) increases could be repeated in the same alveolus by photo exciting a different region of the alveolar wall (Fig. 1C). Hence, uncaging was target specific, and the global [Ca\(^{2+}\)]\(_{cyt}\) increase resulted from conducted Ca\(^{2+}\) responses and not from nonspecific uncaging.

To interpret [Ca\(^{2+}\)]\(_{cyt}\) from fluo 4 fluorescence, we coloaded alveoli with fura 2, which gave a direct readout of the concentration (11). Intra-alveolar injections of the Ca\(^{2+}\) agonist ATP increased [Ca\(^{2+}\)]\(_{cyt}\). Paired determinations in individual alveolar cells indicated the linear relationship between fluo 4 fluorescence and the fura-derived Ca\(^{2+}\) concentration (Fig. 1D). On the basis of this relationship, we estimated that in the targeted region, uncaging increased [Ca\(^{2+}\)]\(_{cyt}\) by 88 \(\pm\) 8 nM (\(n = 5\)).

Photo-excited Ca\(^{2+}\) uncaging in one alveolus caused [Ca\(^{2+}\)]\(_{cyt}\) increases in cells of neighboring alveoli (Fig. 2A). The maximum Ca\(^{2+}\) increase at different distances from the uncaging site correlated linearly (Fig. 2, A and B). To deter-
mine the directionality of Ca$^{2+}$/H$^{11001}$ conduction, we selected cell triads between adjacent alveoli (Fig. 2C). In a cell lying central to two neighboring cells, successive photo excitations of each neighbor could induce similar [Ca$^{2+}$/H$^{11001}$]cyt transients (Fig. 2C, tracings), indicating that the Ca$^{2+}$/H$^{11001}$ conduction was bidirectional.

Alveolar Ca$^{2+}$/H$^{11001}$ conduction occurs through gap junctions. Intercellular communication in alveolar cells occurs through Cx43-containing gap junctions (14), which are inhibited by the Cx43-recognizing peptides gap 26 and gap 27 (7). In gap 26/27-treated alveoli, although photo excitation increased [Ca$^{2+}$/H$^{11001}$]cyt in the targeted cell (Fig. 3, A, arrow, and B, inset), no Ca$^{2+}$/H$^{11001}$ increases occurred in other cells of the alveolus (Fig. 3B), indicating that the peptides inhibited Ca$^{2+}$/H$^{11001}$ conduction. Wash-out of the peptides by an intra-alveolar buffer microinfusion rescued the conduction response (not shown), thereby ruling out toxicity as a possible cause for the inhibition. Peptides corresponding to scrambled sequences in gap 26 and gap 27...
failed to inhibit the conduction (Fig. 3B), indicating that the inhibition resulted from specific sequence recognition on Cx43 and further excluding nonspecific effects of gap 26/27.

Xestospongion C, which inhibits the endoplasmic reticulum receptor for inositol 1,4,5-trisphosphate (11), and PPADS, which is a purinergic receptor blocker (15), each failed to block the stimulated Ca2+ wave conduction (Fig. 3B). However, as expected, xestospongion C and PPADS each blocked ATP-induced Ca2+ increases (Fig. 3C). These findings, taken together with the inhibitions by gap 26/27, indicate that Cx43-based gap junctions and not inositol 1,4,5-trisphosphate or ATP were critical for the present conduction responses.

Alveolar Ca2+ conduction causes type 2 cell secretion. To determine LB exocytosis, we coloaded alveoli with the Ca2+ cage and the LB-localizing dye LTG. As in our previous study (1), LTG-labeled type 2 cells were identified as single brightly fluorescent cells (Fig. 4A, top image). As previously (1), we confirmed the type 2 cell phenotype of LTG-loaded cells by detecting the immunofluorescence of cell-specific surface markers (not shown). Thus nonfluorescent regions of the LTG-loaded alveolus contained type 1 cells.

In alveoli not loaded with the Ca2+ cage, LTG fluorescence of type 2 cells was steady and unaffected by photo excitation (not shown), indicating that the dye was not extruded by nonspecific mechanisms and that it was not photobleached by the uncaging beam. However, in cage-loaded alveoli, photo excitation of the type 1 cell region induced progressive decreases in LTG fluorescence of type 2 cells, indicating stimulation of LB exocytosis (Fig. 4A, bottom images and trace). Similar to our study (1) with intra-alveolar histamine, the fluorescence decreased to 50% of the initial value in 13 ± 2 min (n = 5). These findings are the first direct evidence in alveoli that LB exocytosis results from Ca2+ conduction between type 1 and type 2 cells.

To determine the role of Ca2+ in the maintenance of LB exocytosis, we photo excited single alveoli successively at two separate locations in the type 1 cell region. Although the first photo excitation initiated LB exocytosis, the second photo excitation (given 10 min after the onset of exocytosis) failed to modify the exocytosis rate (Fig. 4B). Hence, we interpret that Ca2+ was the exocytosis-initiating stimulus. However, the failure of the second Ca2+ stimulus to augment the exocytosis rate remains unclear.

Treating alveoli with gap 26/27 blocked the uncaging-induced LB exocytosis, although no inhibition occurred in the presence of scrambled peptides, xestospongion C, or PPADS (Fig. 4C). These results affirm our interpretation that gap junctions provided the primary mechanism for the communicated secretion response. However, as different from the Ca2+ response, which attenuated with radial distance from the photo excitation site (Fig. 2B), exocytosis rates were similar in all responding cells irrespective of the distance from the phototargeted site (Fig. 5, A–C). At distances >100 μm, increasing numbers of cells failed to initiate exocytosis (Fig. 5C). Hence, we interpret that LB exocytosis was initiated by a Ca2+ increase above a stimulation threshold that was evidently not achieved in nonresponding cells. Although we did not determine the threshold directly, based on the calibration against fura 2 (Fig. 1D) and on the distance-attenuated Ca2+ response (Fig. 2B), we estimate that the threshold lay in the 30- to 50-nM range.

Because LTG is pH sensitive, we considered that pH decreases induced by an increase of Ca2+ might cause nonspecific effects on LTG fluorescence (5). However, in a single experiment (not shown) in which we coloaded alveoli with the pH indicator BCECF (5) and the Ca2+ cage, uncaging failed to change the BCECF fluorescence ratio, although an injection of 0.1 N HCl decreased the ratio as expected, indicating that a Ca2+ increase did not modify cell pH (replicated in 3 alveoli).

DISCUSSION

We show here that an increase of [Ca2+]cyt induced at a localized region of an alveolus spreads not only throughout the same alveolus but also to adjacent alveoli, providing the first evidence for the existence of intercellular communication...
among intra-acinar alveoli. Our results were obtained by means of the photo-uncaging approach, which has been widely applied in cultured cells and tissue slices (17, 24) but which we now show to be a viable modality for targeted stimulation in the organ setting. Photo uncaging did not extend beyond the targeted site, thereby ruling out the presence of nonspecific effects in nontargeted regions. The conduction was blocked completely and reversibly by a combination of Cx43-inhibiting peptides but not by peptides containing a scrambled sequence, pointing to Cx43-containing gap junctions as the major route for the interalveolar Ca\(^{2+}\) conduction. It has been reported that Cx43 gap junction channels rectify voltage communication (3). To test this hypothesis, we determined Ca\(^{2+}\) responses in a selected alveolar cell while uncaging Ca\(^{2+}\) in an adjoining cell. Irrespective of the orientation of the uncaged cell with respect to the responding cell, Ca\(^{2+}\) increases in the responding cell were always similar. These findings indicate that alveolar Ca\(^{2+}\) conduction was directionally symmetrical and nonrectified. We point out that our interpretations are limited to a two-dimensional analysis of Ca\(^{2+}\) conduction, although the Ca\(^{2+}\) spread was probably three dimensional. Nevertheless, our findings constitute the first direct evidence that Ca\(^{2+}\) communication exists among adjoining pulmonary alveoli.

After the Ca\(^{2+}\) uncaging, LB exocytosis occurred in both the photo-targeted alveolus as well as in the nontargeted alveolus. We estimate that at the uncaging site, Ca\(^{2+}\) increased by an order of 80–90 nM, namely, within the range of alveolar Ca\(^{2+}\) increases elicited by physiological challenges (1, 25). Our previous findings, in particular that physiological stimuli induce synchronous Ca\(^{2+}\) oscillations among alveolar cells, indirectly suggested that Ca\(^{2+}\) conduction between adjacent alveolar cells underlies the initiation of LB exocytosis (1). Here, we reaffirm this mechanism, because, within the same alveolus, Ca\(^{2+}\) increases in photo-targeted type 1 cells initiated LB exocytosis in nontargeted type 2 cells. Furthermore, anti-Cx43 peptides inhibited the exocytosis, providing the first in situ evidence that the secretion stimulus was conducted from type 1 to type 2 cells across Cx43-containing gap junctions. The exocytosis response reaffirms previous reports from our (1) and other (9, 26) laboratories demonstrating that an increase of type 2 cell Ca\(^{2+}\) is the critical stimulus for LB exocytosis, although Ca\(^{2+}\)-independent exocytosis involving PKC- or cAMP-dependent mechanisms might also occur (18, 23). In cultured type 2 cells, a [Ca\(^{2+}\)]\(_{cyt}\) increase induced by ionophores, cell stretch, or secretagogues (9, 26) or by uncaging methods (8) stimulates surfactant secretion. These findings are consistent with the notion that in regulated exocytosis, vesicle docking at the cell membrane is followed by a final Ca\(^{2+}\)-dependent step that activates the release of vesicular contents.

Our findings address the Ca\(^{2+}\) role after the initiation of exocytosis. In uncaging experiments, the magnitude of the conducted Ca\(^{2+}\) increase decreased with distance from the uncaging site. Because graded, Ca\(^{2+}\)-dependent surfactant secretion occurs in cultured alveolar epithelial cells (23, 26), we expected the exocytosis rate to decrease as Ca\(^{2+}\) levels decreased along the route of Ca\(^{2+}\) conduction. However, the rates were more or less similar in all responding cells, indicating that at the Ca\(^{2+}\) levels encountered in these cells, exocytosis was Ca\(^{2+}\) insensitive. In other experiments, we uncaged NP-EGTA two times in the same alveolus such that the Ca\(^{2+}\) increase due to the first uncaging had dissipated and Ca\(^{2+}\) levels were baseline before the second uncaging. In these experiments, although the first Ca\(^{2+}\) increase stimulated LB exocytosis, the second had no effect, indicating that sequential Ca\(^{2+}\) increases did not additively augment exocytosis. Despite the recognized
role of Ca\(^{2+}\) in the initiation of surfactant secretion, we suggest that after the initiation step, secretion might be maintained by processes that no longer require elevations in [Ca\(^{2+}\)]\(_{cyt}\).

The proposed mechanisms of intercellular Ca\(^{2+}\) conduction implicate gap junctional or paracrine mechanisms (2, 10). Evidence for gap junctional communication is commonly obtained by the application of inhibitors such as heptanol, halothane, and \(\alpha\)-glycyrrhetinic acid, which have complicating nonspecific effects (7, 14). The knockout approach is also problematic because, for Cx43, which is a major alveolar connexin (14), gene-targeted deficiency is incompatible with postnatal survival (21). The advantage of the present connexin-recognition peptides is that these peptides block gap junctional communication in several cell types (4, 7, 14, 16), including cultured alveolar cells (12, 13), by recognizing specific sequences in extracellular loops 1 and 2 of Cx43. The extracellular interaction eliminates nonspecific effects of cellular uptake while identifying the specific connexin responsible for the gap junctional conductance. We confirmed that the inhibitory effect is reversible, thereby ruling out the presence of cell toxicity induced by the peptides. We conclude that the combined application of gap 26 and 27 peptides provides an effective means for blockade of gap junctional communication in intact pulmonary alveoli. Our findings point to interalveolar communication as a novel mechanism that requires further consideration in the context of lung disease.

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