Sharing signals: connecting lung epithelial cells with gap junction channels

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Koval, Michael. Sharing signals: connecting lung epithelial cells with gap junction channels. Am J Physiol Lung Cell Mol Physiol 283: L875–L893, 2002; 10.1152/ajplung.00078.2002.—Gap junction channels enable the direct flow of signaling molecules and metabolites between cells. Alveolar epithelial cells show great variability in the expression of gap junction proteins (connexins) as a function of cell phenotype and cell state. Differential connexin expression and control by alveolar epithelial cells have the potential to enable these cells to regulate the extent of intercellular coupling in response to cell stress and to regulate surfactant secretion. However, defining the precise signals transmitted through gap junction channels and the cross talk between gap junctions and other signaling pathways has proven difficult. Insights from what is known about roles for gap junctions in other systems in the context of the connexin expression pattern by lung cells can be used to predict potential roles for gap junctional communication between alveolar epithelial cells.

connexin; calcium; surfactant; signal transduction; oxidant injury

AS A CLASS OF MEMBRANE CHANNELS, gap junctions are unique and complex. Gap junction channels interconnect cells by forming a direct link to enable the diffusion of small aqueous molecules and ions from one cell to its nearest neighbor. This enables both the flow of specific intercellular signals and metabolic cooperation between communicating cells in a tissue. This review will cover potential roles for gap junction proteins (connexins) as they are expressed by alveolar epithelial cells and which are applicable to pulmonary epithelial cells in general. Some aspects of gap junctions related to vascular endothelial function will be addressed; however, there are a number of recent reviews that cover roles for connexins in the cardiovascular system (29, 170, 174, 240, 241, 245). The interested reader is also referred to other recent general reviews referenced in the text and a recent monograph that covers aspects of gap junctional communication beyond the scope of this review (122).

Nearly every mammalian cell expresses connexins, and cells relevant to pulmonary physiology are no exception. On the basis of the human and murine genomes, there are almost two dozen mammalian connexins (Fig. 1), which are differentially expressed, depending upon cell phenotype. The nomenclature most typically used, which will be used here, is to denote a specific connexin by “Cx” plus a number corresponding to the predicted molecular mass based on the amino acid sequence. This method leads to some difficulties, particularly when comparing connexin orthologs between species. For instance, human/murine Cx46 is the ortholog to chick Cx56 and human/murine Cx50 is the ortholog to chick Cx44. Also, by SDS-PAGE, connexins tend to migrate at a higher relative molecular mass ($M_r$) than the predicted $M_r$ and frequently migrate as a set of bands based on phosphorylation state, so the molecular mass denoted by the name can be somewhat misleading. An alternative connexin nomenclature based on sequence homology referring to α- and β-connexin subgroups is occasionally used (153), and other alternative connexin numbering schemes have also been proposed (33).

A single gap junction channel consists of two hexameric hemichannels, one in each cell, which are formed in intracellular compartments and then transported to the plasma membrane, where they dock together to form the complete channel. The formation of connexin...
hemichannels is complex and appears to depend upon the connexin examined. For instance, Cx32 hemichannels are most likely formed in the endoplasmic reticulum (ER) (60, 152), as is the case for most multimeric transmembrane proteins (118). However, other connexins, such as Cx43 and Cx46, are assembled in aspects of the Golgi apparatus/trans-Golgi network (TGN) (59, 149, 158, 197, 306). Connexin hemichannel formation is a regulated process, since cells control whether compatible connexins intermix to form hemichannels (59, 149). Trafficking of connexin hemichannels to the plasma membrane is also regulated, which enables cells to regulate gap junctional communication by increasing or decreasing the number of channels interconnecting cells (13, 51, 121, 214, 239, 263, 279).

In addition to intrinsic channel activity at the plasma membrane, connexins also associate with cofactors, such as zonula occludens (ZO)-1 (15, 95, 143, 156, 266), calmodulin (215, 291), and tubulin (96), which have the potential to modulate their function. There is increasing evidence suggesting that gap junctions and tight junction proteins are biochemically associated (146, 147), which is underscored by the observation that ZO-1 interacts with both gap junction and tight junction proteins (64, 189). The response of gap junctions is also modulated by cystic fibrosis transmembrane conductance regulator (46–48), which may be part of the junctional complex (220, 248), and by wnt-1 (5, 276), suggesting a link with β-catenin and cadherins in regulation gap junctions (196, 302). However, the extent of cross talk between transmembrane proteins at the junctional complex is not well understood at present.

What, then, are the roles for connexin heterogeneity? The answer to this question is at least threefold. First, different connexins have different selectivity; that is, they permit different classes of molecules to diffuse through the channels. Second, different connexins have the capacity to specifically intermix, which may influence which cells communicate with their neighbors based on whether or not the combinations of connexins expressed by the cells are compatible. Third, different connexins have different gating characteristics, in that they are differentially opened and closed in response to changes in membrane potential, cytoplasmic pH, and connexin phosphorylation state. Note that the ability of connexins to intermix also enables cells to create gap junction channels with unique permeability and gating characteristics distinct from channels composed of a single connexin. Also, an implication of connexin gating is that connexin expression alone does not control the level of gap junctional communication; instead cross talk between connexins and other signaling pathways also needs to be considered.

DIFFERENT CONNEXINS, DIFFERENT PERMEABILITY

Gap junction channels are not nearly as selective as ion channels, where ions have distinct coordination states with specific amino acids (76, 77). Instead, the selectivity of gap junction channels is relative; that is, gap junction channels composed of different connexins show different rates of diffusion for a given molecule, rather than absolute differences in molecular permeability. Taking into account the possibility that connexins can have multiple conductance states depending on the cell system examined, one finds that determining the precise signals moving through a gap junction channel at any given time is extremely difficult.

To be precisely quantitative, it is best to compare gap junctional transfer of multiple tracers for the same system. One of the best approaches is to measure perme selectivity, where the net ionic flux is measured electrophysiologically simultaneously with fluorescent dye transfer measured by fluorescence microscopy (40, 275, 280). This provides a means for the investigator to normalize for total gap junction channel cross-sectional area, while at the same time measuring permeability of dyes with distinct mass and charge (80, 204). Other approaches include measuring simultaneous transfer of multiple dyes (148, 204) and/or radiolabeled metabolites (100).

A good illustration of the complexity of determining connexin permeability is to compare Cx32 with Cx43, both of which are expressed by alveolar epithelial cells (2, 3, 131, 169). Negatively charged, fluorescent dyes such as calcein, LY, or the Alexa series of dyes (Molecular Probes, Eugene, OR) are more readily transferred through Cx32 gap junction channels than Cx43 channels, which gives the initial impression that Cx32 is more permeable than Cx43. However, an examination of metabolite transfer indicates that ATP/ADP diffusion is ~10-fold faster through Cx43 channels compared with Cx32 (100, 204). Conversely, glutathione flux through Cx32 and Cx43 channels is roughly comparable. Thus when considering whether or not cells are coupled, one needs to consider both the connexin expression pattern and the probe or metabolite examined.
CONNEXIN COMPATIBILITY

Connexin intermixing is classically thought of in terms of heterotypic interactions between connexins expressed by different cells and heteromeric interactions between connexins expressed by the same cell (Fig. 2). On the basis of studies of connexin intermixing, described below, it seems likely that heteromeric gap junctions may be fairly common in vivo. However, this is not always the case. For instance, hepatocytes express both Cx26 and Cx32 and show both heteromeric and homomeric gap junction channels, as determined by scanning transmission electron microscopy (254). In contrast, purely heterotypic gap junctions are likely to be less frequent, although heterotypic coupling is suggested by systems such as astrocytes and oligodendrocytes, which communicate yet express distinct connexins (199).

Connexin intermixing has been examined functionally (by measuring channel activity), biochemically (co-immunopurification and cross-linking), and by immunogold and immunofluorescence microscopy. The rules governing connexin intermixing initially appeared to correspond to amino acid homology grouping as shown in the dendrogram (Fig. 1). However, this has turned out not to be the case, particularly for heterotypic interactions, since these interactions are sensitive to subtle changes in the sequence of the second extracellular loop domain often missed by normal amino acid homology comparisons. For instance, Cx40 and Cx43 have the capacity to form heteromeric gap junction channels (38, 55, 274, 275), but Cx43 hemichannels cannot pair with Cx40 hemichannels to form functional gap junctions (34, 80, 119, 296). Cx46 represents another exception to the normal restrictions in connexin compatibility, since it can apparently form heterotypic channels with almost every connexin (293, 296), whether or not it can form heteromeric channels with them (59).

One consequence of heterotypic gap junction formation is that that cells can form gap junction channels with unique permeability characteristics not obtainable with a gap junction channel composed of a single connexin. For instance, Cx26 has a fairly restrictive permeability (40, 204) and forms heteromeric gap junction channels with Cx32 (4, 21, 85, 175, 256). Cx32 is fully permeable to cAMP and cGMP; however, on the basis of assays of reconstituted channels, Cx32/Cx26 heteromers are preferentially permeable to cGMP compared with cAMP (21), indicating that the stoichiometry of Cx26 to Cx32 enables cells to fine tune metabolic coupling through these channels. There are other examples of heteromeric gap junction channels that have unique permeability characteristics, including Cx37/Cx43 (30, 164), Cx40/Cx43 (38, 55, 274, 275), and Cx43/Cx45 (148, 191).

Cx32 and Cx43 represent the one of the “best” examples of incompatible connexins, on the basis of biochemical data (4, 59, 84, 85, 113) and the inability of Cx32 hemichannels to pair with Cx43 hemichannels based on dye transfer measurements (80, 296). Electrophysiological measurements from transfected Xenopus oocytes suggest that Cx32 and Cx43 may form some low-conductance heterotypic channels (283), although this point is controversial. Cx26 and Cx43 also are incompatible, according to dye transfer measurements (80, 265, 296). However, tumor cells transfected with Cx26, but not Cx32, communicate with vascular endothelium, which expresses Cx43 and apparently no Cx26-compatible connexins (135). Mutant forms of Cx26 associated with the skin disease palmoplantar keratoderma also have the capacity to act as dominant-negative inhibitors of Cx43 (232). Clearly, some care is needed when one is considering, on the basis solely of connexin expression pattern, whether or not cells are able to communicate through gap junctions.

OPEN AND SHUT: GATING CONNEXIN CHANNELS AND REGULATION OF CELL GROWTH

Cells use differential gating to specifically reduce or increase gap junctional communication by changing the open probability for a subset of connexins. For instance, Cx43 and Cx46 are gated at acidic pH, whereas Cx32 is relatively insensitive to pH (88, 173). At acidic pH, Cx43 and Cx46 are gated by a ball-and-chain mechanism (79, 193). In fact, this mechanism will still function when a truncated version of Cx43 is coexpressed with a soluble form of the Cx43 COOH terminus (193).

Differential phosphorylation is another possible mode for specifically attenuating a subset of connexins. Based on consensus sequences, it is likely that most connexins are phosphorylated. Cx32, Cx43, and Cx46 are among the connexins that have been shown to be phosphorylated (104, 161), although Cx26 is not (19, 310). Functionally, the effect of phosphorylation on Cx43 function has been best characterized. Kinase cascades linked to cell growth control have been shown to attenuate Cx43 gap junction channels by decreasing connexin open channel probability, e.g., “closing” gap junction channels (125). For instance, phorbol esters stimulate protein kinase C, which inhibits Cx43 as-
Assembly and thus Cx43-mediated gap junctional communication (160). This correlated well with changes in Cx43 phosphorylation that occurred during mitosis (160, 301), where Cx43 gap junction channels are dis-assembled as well. Phosphorylation of Cx43 by pp60-src has been found to decrease gap junctional communication and correlates with a lack in growth control (163, 176, 313). MAP kinase signaling pathways have also been implicated in Cx43 phosphorylation to inhibit channel permeability (126, 285, 288, 289) and may be the pathway activated by the gap junction inhibitor methanandamide (65).

Consistent with a role for gap junctions in growth control, many agents that inhibit gap junctions are also frequently tumor promoters (231, 269). For instance, tobacco smoke condensates contain substances with the capacity to inhibit gap junctional communication (123, 177, 183, 184, 236, 237). Cell lines derived from lung tumors are also frequently not coupled through gap junctions, which can be due to a lack of connexin expression or due to constitutively closed gap junction channels (43, 233, 234). However, not all correlations of changes in connexin expression with tumorigenesis are significant, since reports that Cx37 mutations might be associated with lung cancer (178) were later found to reflect allelic variability in the human Cx37 gene (150).

On the other hand, agents that enhance gap junctional communication may confer growth control to cells (13, 120). Also, forced connexin expression can sometimes reduce the tumor-forming capacity of some cell lines (101, 233). There may be a role for connexin specificity here, since Cx32 suppressed the tumor-forming capacity of a lung carcinoma cell line (234), although Cx43, but not Cx32, conferred growth control to a glioma cell line (101). As a further complication to understanding roles for gap junctions in carcinogenesis, heterocellular communication between tumor cells and endothelial cells appears to be required for transmigration (135). In other words, uncontrolled growth is correlated with lack of coupling, but coupling may be required for metastasis.

CONNEEXIN-DEFICIENT MICE AS MODELS FOR DISEASE

There have been a number of human diseases traced to connexin mutations, and transgenic connexin-deficient mouse models frequently mimic these conditions (35, 145, 250, 295, 297). Given the multimeric nature of gap junction channels, mutant connexins frequently are autosomal dominant; that is, they exert a dominant-negative effect on native connexins (59, 155, 258, 304) and thus have the potential to affect the function of more than one class of wild-type connexin (227, 232). This should be kept in mind when comparing human diseases to mouse models where connexin expression is absent.

Cx43-deficient mice have the phenotype most directly relevant to pulmonary physiology. Transgenic mice lacking Cx43 are born cyanotic and die soon after birth (222). The mice develop pulmonary edema primarily from reduced cardiac output, since the hearts of these mice are malformed and have outflow tract defects. However, this is not a direct effect of Cx43 deficiency, since Cx43 expression in the normal outflow tract is low. Instead, the defect is due to improper neural crest cell migration during embryogenesis, which does not support normal heart development (174). Consistent with a role for Cx43 in human heart development, human Cx43 mutations have also been linked to visceroatrial heterotaxy (VAH) and pulmonary stenosis (31), although not all VAH patients express mutant Cx43 (92). Mice expressing a dominant-negative Cx43 construct show abnormal heart development comparable to Cx43 −/− mice (258). Interestingly, mice overexpressing Cx43 also show a similar phenotype, suggesting that too little or too much gap junctional communication can be detrimental, since too little Cx43 activity causes neural crest cell migration to be uncoordinated and too much Cx43 inhibits cell migration altogether (52, 128).

To determine whether another connexin could substitute for Cx43, Plum et al. (219) created “knock-in” mice, in which the Cx43 gene was replaced with either Cx32 or Cx40. In either case, the neonatal lethal phenotype was rescued, a so-called shared function for connexins, consistent with redundancy in the connexin gene family. However, Cx32 knock-in mice had subtle heart malformations, and both knock-ins were susceptible to cardiac arrhythmia, reflecting unique functions for Cx43. As another example for connexin specificity in transgenic mice, replacement of Cx50 with Cx46 corrected the cataract abnormality associated with Cx50 deficiency (292, 294). However, lens growth remained abnormal, suggesting a unique function for heteromeric Cx46/Cx50 gap junction channels in this process (292).

The neonatal lethal phenotype of Cx43-deficient mice has made it difficult to elucidate other physiological roles for Cx43, such as direct roles in lung physiology. Studies of Cx43 +/+ heterozygous mice have elucidated roles for Cx43 in cardiac conduction (112) and bone development (167) and may be useful for studies related to the lung. Another approach has been to use cre recombinase to specifically excise Cx43 engineered to be flanked by lox-P sites from a subset of tissues (309). Liao et al. (172) used cre expression driven by the Tie-2 promoter to preferentially excise the Cx43 gene from endothelial cells. These mice develop normally, and their endothelial cells express two other connexins, Cx37 and Cx40, but they have abnormally low blood pressure and a lowered heart rate. Lowered blood pressure was due to increased plasma nitric oxide (NO) production, most likely due to increased endothelial nitric oxide synthase activity. However, because endothelial NO production is linked to gap junctional coupling between vascular smooth muscle and endothelial cells (73), this indicates that Cx43 depletion actually enhanced coupling between these cells. Interestingly, Cx40-deficient mice have increased blood pressure and decreased coupling, indicating a predominant role for Cx40 vs. Cx43 in inter-
connecting vascular smooth muscle and endothelium (62). This also underscores the concept of different roles for different connexins.

CALCIUM TRANSIENTS AND HETEROCYTOCELLULAR REGULATION OF SURFACTANT SECRETION

The alveolar epithelium is a heterogeneous monolayer consisting of at least two cell types, type II cells and type I cells, which are in direct contact (56, 74, 141, 272). Type I cells make up >90% of the alveolar epithelial surface area, which is consistent with their role as the site of gas exchange between the atmosphere and capillary blood. However, there are roughly twice as many type II cells as there are type I cells. Assuming that both cell types are uniformly distributed throughout the alveolar epithelium, this suggests that nearly all type I cells are likely to be in direct contact with at least one type II cell, as well as with other type I cells. Freeze-fracture electron microscopy has shown that alveolar epithelial cells are interconnected by gap junctions (42, 105, 243).

Type II cells produce pulmonary surfactant, a protein-lipid complex required to lower the surface tension of the lung air-water interface to prevent lung collapse and to facilitate expansion (63, 179). Surfactant production is a regulated process that can be stimulated by a range of hormones and pharmacological agents. However, given the architecture of the alveolus, the notion that regulation of surfactant secretion involves both type II and type I alveolar epithelial cells is very appealing.

Hyperventilation, by increasing either ventilation frequency or tidal volume, has been shown to increase pulmonary surfactant secretion, both in the intact animal and in the isolated perfused lung (182, 202, 203, 210). Because hyperventilation can stimulate surfactant secretion, this has led to studies examining the effect of mechanical stimulation on isolated type II cells cultured on deformable substrata. Subjecting type II cells to a single, transient tonic mechanical stretch, which increased surface area by ~15–25%, increased surfactant lipid secretion by a factor of three (299). This was accompanied by a short transient increase in intracellular calcium, suggesting that the increase in surfactant secretion was due to calcium-mediated signal pathways. The existence of calcium-regulated pathways for surfactant secretion stems from a large body of pharmacological evidence obtained from primary type II cells in culture. Increases in intracellular calcium increase surfactant secretion either by a direct effect on the secretory machinery (70, 75, 109, 117, 242, 316) or through a protein kinase C-dependent pathway (44, 108, 224, 229, 286), or both. Stimulation of surfactant secretion by endothelin-1 also shows both a calcium- and protein kinase C-dependent component (244).

Although it is clear that direct mechanical stimulation can enhance type II cell secretion, whether this occurs in situ is still an open question. For instance, during normal breathing, the changes in lung volume generally correspond to relatively small changes (~5%) in alveolar surface area (97, 270), although during sighs or yawns, alveolar surface area may increase by ~10–15% (299). Also, stress analysis of alveoli suggests that type II cells, which are localized to “corners,” tend to be shielded from stress forces sensed by other alveolar cells (93). Because type II cells tend to be more sensitive to mechanical trauma than type I cells (271), it might be advantageous for type I cells to have a mechanical sensor function.

To directly address whether type I cells play a role in surfactant secretion, Ashino et al. (11) have used the technically challenging technique of whole lung vital fluorescence microscopy. These investigators isolated perfused rat lungs labeled with calcium-sensitive dyes to study the transmission of calcium-mediated signal pathways to type II cells, which was correlated to secretion. They found that 15 s of hyperinflation initiated signals in type I cells as opposed to type II cells. Hyperinflation caused type I cells to generate cytosolic calcium oscillations, which, in turn, were transmitted to type II cells. Type II cell secretion was followed fluorometrically using lamellar bodies labeled with LysoTracker green, which is released by secretion. They found a correlation between the extent of LysoTracker green secretion and the frequency of calcium oscillations, suggesting a functional relationship between the two processes. Also, cytosolic increases in type II cell calcium and LysoTracker green secretion were inhibited by halothane and heptanol, agents that preferentially block intercellular communication through gap junctions. The use of two different inhibitors is important here, because heptanol has been shown to affect purinergic receptor activity (111), and L-type calcium channels are partially inhibited by halothane (205). Thus this provided the first in situ evidence suggesting that heterocellular type II-type I interactions in general and gap junctions in particular are important for regulating surfactant secretion by controlling cytosolic calcium.

Molecular evidence corroborating this finding would be welcome, particularly because it appears likely that other signaling pathways might play a role in regulating surfactant secretion in addition to gap junctions. Nonetheless, the notion that heterocellular interactions are important for regulating surfactant secretion also suggests that disrupting type I-type II cell interactions might affect surfactant turnover. Consistent with this, surfactant abnormalities are frequently associated with diseases such as acute respiratory distress syndrome (ARDS) and ventilator-induced injury (17, 78, 106, 281), where cell-cell contacts may be disrupted (140, 154, 180, 272, 315).

CROSS TALK BETWEEN COMMUNICATION PATHWAYS: CALCIUM WAVES

Transmission of intercellular calcium transients, or calcium waves, can occur through two predominant mechanisms: using gap junction channels or by paracrine stimulation of purinergic receptors through ATP release. It seems likely that both processes can occur in
alveolar epithelial cells (Fig. 3). As indicated above, in situ microscopy supports a role for heterocellular type II-type I gap junctions in transmitting mechanically induced calcium waves. Gap junctional transmission of calcium (211, 238) and inositol trisphosphate (89, 238) have been identified as elements that participate in calcium wave transmission. In particular, HeLa cells transfected with enhanced green fluorescent protein-tagged Cx43, Cx32, or Cx26 have been used with the calcium-sensitive dye fura 2 to image calcium flux through gap junction channels in response to mechanical stimulation (211). On the basis of studies using small connexin peptides as gap junction inhibitors (18, 24, 83), gap junctional communication has also been implicated in propagating mechanically induced calcium waves in airway epithelium (23) and, relevant to stimulated surfactant secretion, between alveolar epithelial cells cultured to have a type I-like phenotype (131).

In many of the systems mentioned above, paracrine ATP stimulation also can mediate calcium waves, including HeLa cells (211) and airway epithelial cells (82). Calcium waves by paracrine stimulation of purinergic receptors have also been demonstrated for osteoblastic cells (137, 138) and insulin-secreting cells (39). It is likely that stimulated ATP secretion is a universal feature of most cell types in response to even mild mechanical stimulation (208). Stimulated UTP secretion can also initiate calcium waves (32, 124). Most instances of calcium waves generated by paracrine ATP secretion are due to stimulation of P2Y-type receptors.

Type II cell responses to purinergic agonists have been well characterized. Initial identification of type II cell purinergic P2Y2 receptor activity and calcium-stimulated surfactant secretion was determined pharmacologically (98, 226). The high, equivalent activity of ATP and UTP in stimulating surfactant secretion is particularly strong evidence for P2Y2 receptors, which have been cloned from type II cells (225). However, because type II cells had EC50 in the range of 0.5 μM for β,γ-CH2-ATP (98), this also raises the possibility that alveolar epithelial cells express P2X-type purinergic receptors, because P2X-type receptors are insensitive to this agonist at this concentration range (206). Consistent with this notion, an alveolar epithelial cell line, A549 cells, expresses P2X4 in addition to P2Y2 receptors (311). This is also complicated by other modes for regulating intracellular calcium, such as L-type voltage-gated calcium channels that may also be involved in surfactant secretion (67, 244).

Consistent with type II cell purinergic receptor activity, mechanically induced calcium waves were transmitted between cultured alveolar epithelial cells with a type II-like phenotype using extracellular ATP release and purinergic receptor stimulation and were not affected by peptide gap junction inhibitors (132). Whether ATP release regulates surfactant secretion in vivo remains to be determined. In particular, it is worth noting that a monolayer of type II-like cells in direct contact is distinct from the heterocellular monolayer found in the alveolus in situ. Intriguingly, Isakson et al. (131) found that type I-like alveolar epithelial cells preferentially used gap junctional communication, instead of purinergic receptor stimulation, to transmit mechanically induced calcium waves. Given this and the studies of Ashino et al. (11), it seems plausible that regulating transmission of calcium transients between type I and type II cells may involve a combination of gap junctions and paracrine ATP secretion (Fig. 3). This may also involve feedback communication from type II cells to type I cells. Consistent with this possibility, we have found that type I-like cells show transient increases in intracellular calcium in response to extracellular ATP (P. Ruckert and M. Koval, unpublished results) and thus might respond to ATP secreted by type II cells in situ.

In addition to calcium-mediated stimulation of surfactant secretion, agents that stimulate cAMP formation, such as β-agonists (e.g., epinephrine, terbutaline, isoproterenol), adenosine and adenosine analogs [5'-(N-ethylcarboxamido)adenosine], and other agents (e.g., forskolin), enhance surfactant secretion through protein kinase A-dependent pathways (71, 99, 107, 186, 286, 287). Because secretagogues that act through calcium-mediated and cAMP-mediated pathways are additive, this suggests that these are multiple distinct pathways used by type II cells to regulate surfactant secretion. This may also tie in with gap junctional communication, since cAMP transmission through gap junction channels has been clearly established for other cell systems (21, 166) and may very well be transmitted from type I cells to type II cells. Hetero-

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**Fig. 3.** Potential communication pathways between alveolar epithelial cells to control surfactant secretion. Gap junctional transmission (1) of either calcium or inositol trisphosphate (IP3) generates increased cytosolic calcium in type II cells, which, in turn, could stimulate surfactant secretion either through protein kinase C activation or through a direct effect of calcium on lamellar body fusion. Increased cytosolic calcium can also be mediated through secretion of either ATP (2) or UTP acting on P2Y2 purinergic receptors. Protein kinase A activation through stimulation of A2 purinergic receptors by ATP can also occur (3). Other potential receptors (e.g., β-adrenergic receptors), cAMP transmission through gap junctions, direct stimulation of type II cells, potential feedback loops, potential purinergic receptors on type I cells, and interactions between homologous cells are omitted from this diagram. It seems likely that multiple communication pathways between alveolar epithelial cells act in concert to regulate surfactant secretion.
cultures of alveolar epithelial cells with mixed phenotype should be useful to determine whether this is the case (see INTERCELLULAR COMMUNICATION PATHWAYS IN THE ALVEOLUS).

CONNEXIN HEMICHANNELS AS PLASMA MEMBRANE CHANNELS

As a further complication, connexin hemichannels may play a role in ATP secretion. Hemichannels are connexin hexamers at the plasma membrane that are not coupled to adjacent cells that have the capacity to act as membrane channels. Connexin hemichannels can be opened experimentally by chelating extracellular calcium (171). Recent studies suggest that expression of Cx32 and Cx43 transfected into a number of cell types enhances ATP secretion through a pathway that does not require membrane trafficking and is inhibited by gap junction inhibitors (54). This has led to the suggestion that connexin hemichannels at the cell surface might mediate ATP release directly from the cytosol to the extracellular environment (54, 257). Permeability from connexin hemichannels, by and large, seems to reflect the permeability of complete gap junction channels and thus could certainly enable ATP diffusion (88, 171, 268, 282). However, it is still feasible that cells might use a different plasma membrane channel for mediating ATP release that nonetheless requires cell-cell coupling through gap junctions.

Another potential role for connexin hemichannels is suggested by studies that show that metabolically stressed astrocytes have connexin-dependent permeability to fluorescent tracers based on cells expressing Cx43 antisense mRNA (53). Given this, connexin hemichannels may release ATP as part of an apoptosis pathway. Consistent with this possibility, Cx46 hemichannels open under physiological conditions (213, 268, 282), an intriguing notion given the upregulation of Cx46 in response to lung injury (described below). The notion of functional roles for connexin hemichannels is being further explored, as this is still a controversial area in need of further study.

REGULATED CONNEXIN EXPRESSION BY THE ALVEOLAR EPITHELIUM

Isolated type II alveolar epithelial cells in culture show many characteristics of type II cells in vivo, such as surfactant production (69). However, type II cells are also exquisitely sensitive to culture conditions, and the rapid “dedifferentiation” of type II cells has been a complicating factor in using cultured cell models (212). Methods to manipulate alveolar epithelial cell phenotype have been developed by many researchers and have involved many different approaches, including low serum (26), keratinocyte growth factor (KGF) (1, 27, 36, 132, 246, 303), different extracellular matrix (ECM) substrata (1, 8, 36, 58, 115, 132, 303), coculture with fibroblasts (110, 246), and air-liquid interfaces (7, 72, 303). Phenotype markers for alveolar epithelial cells include using surfactant protein production as a readout for the type II phenotype and markers such as aquaporin 5 (181) and T10 (298) for the type I phenotype.

Isolated alveolar epithelial cells can be cultured to dedifferentiate toward a type I phenotype that shares some characteristics with bona fide isolated type I cells (68, 136) and type I cells in situ (2, 181, 298). Rigorously defining the phenotype of cultured alveolar epithelial cells will require analysis of total mRNA expression using DNA array technology. However, when used with caution and when interpreted in parallel with studies from intact animals, cultured alveolar epithelial cells can offer some insights into the function of alveolar epithelial cells in vivo.

Highlighted in the dendrogram (Fig. 1) are six connexins shown to be expressed by alveolar epithelial cells at the level of mRNA: Cx26, Cx32, Cx30.3, Cx37, Cx43, and Cx46 (see also Table 1). By immunofluorescence, alveolar epithelial cells also express Cx40 (131, 132). Although this was done using antibodies known to cross-react with Cx43 (shown by Severs et al. in Ref. 245), the cells in some instances did not express Cx43 (132). Using RT-PCR, we did not detect Cx40 mRNA expression by freshly isolated or cultured alveolar epithelial cells (1), which may reflect cell phenotype. Other connexins not amplified from alveolar epithelial cell mRNA by RT-PCR were Cx30, Cx31, Cx31.3, Cx33, Cx45, or Cx50 (1), again with the caveat that the absence of a given connexin could be a function of the differentiated state of the cells examined.

With respect to connexins, freshly isolated type II cells show high levels of Cx32 expression (1, 169), consistent with type II cells in situ (2). Expression of Cx32 can also be preserved by culturing cells under conditions in which the type II cell phenotype is preserved, such as using medium containing KGF (1, 132). By and large, when cultured under conditions where the alveolar epithelial cells lose type II cell characteristics and gain type I cell characteristics, the cells downregulate Cx32 expression to undetectable levels.

### Table 1. Connexin expression by AEC

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Cx32</th>
<th>Cx26</th>
<th>Cx43</th>
<th>Cx46</th>
<th>Surfactant Proteins</th>
</tr>
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<tr>
<td>Type II AEC in situ</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+/−</td>
<td>yes</td>
</tr>
<tr>
<td>AEC in culture + KGF</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>yes</td>
</tr>
<tr>
<td>Bleomycin-injured lung, AEC in culture + fibronectin</td>
<td>−</td>
<td>−/*</td>
<td>++</td>
<td>+</td>
<td>no</td>
</tr>
<tr>
<td>Type I AEC in situ</td>
<td>−</td>
<td>−</td>
<td>++</td>
<td>−</td>
<td>no</td>
</tr>
<tr>
<td>AEC in culture + laminin</td>
<td>−</td>
<td>+</td>
<td>+/−</td>
<td>+</td>
<td>variable</td>
</tr>
</tbody>
</table>

Protein expression for selected connexins (Cx) and surfactant proteins by alveolar epithelial cells (AEC) with different phenotypes. KGF, keratinocyte growth factor. *Cx26 expression has not been determined for bleomycin-injured lung.
with within 24 h (1, 169). However, Cx32 expression is not tightly linked to the type II phenotype, as there are some instances where cultured cells express surfactant protein C but not Cx32 (132).

The expression of the other connexins by cultured alveolar epithelial cells is more complex and depends upon the surface that the cells are cultured on. When cultured on tissue culture plastic, with serum-containing medium, there is a reduction of Cx26 to ~40% of type II cell levels and transient increases in Cx43 and Cx46 expression, which peak at about days 3–4 of culture (1, 169). The level of Cx43 expression is proportional to the level of fibronectin deposition by the cultured alveolar epithelial cells (8, 259), suggesting that ECM has a role in upregulating Cx43 expression. Interestingly, culture of alveolar epithelial cells with blocking antifibronectin antibodies caused a shift in Cx43 localization from a membrane associated to a cytosolic form (115). A comparable accumulation of cytosolic Cx43 has been found to occur when cells are treated with proteosome inhibitors that block ER-associated degradation pathway (198, 278, 279). An intriguing possibility is that the redistribution of Cx43 to the cytosol induced by disrupting alveolar cell-matrix contacts might indicate that gap junctions are involved in regulating alveolar cell stress responses (157) or detachment-induced apoptosis (anoikis) (6).

In contrast to a fibronectin-enriched ECM, laminin-rich ECM induced alveolar epithelial cells to produce less Cx43 and more Cx26 (115, 132). Laminin alone was not sufficient to induce Cx32 expression; instead, KGF was also required for Cx32 upregulation (132). This difference in alveolar epithelial cell response to ECM composition is intriguing, because remodeling from a laminin-enriched matrix to a fibronectin-enriched ECM may accompany the re-epithelization of a denuded lung basement membrane (9, 221, 228, 230). Note that the reaction of alveolar epithelial cells to different ECM components is not universal for all cells, since keratinocytes upregulate Cx43 expression in response to laminin but not fibronectin (162).

On the basis of immunohistochemistry of whole lung sections, it appears that Cx32 is expressed exclusively by type II alveolar epithelial cells in normal adult rat lung (2). Cx43 is fairly ubiquitous (2, 142) and likely to be the major connexin interconnecting type II and type I cells (2). Cx26 is expressed to varying extents, most consistent with a type II localization in the adult ferret (41, 42), and consistent with the notion that type II cells have heteromeric Cx26/Cx32 channels. Cx26, Cx32, and Cx43 are also expressed by airway epithelial cells (22, 24, 28, 42). Expression of Cx37 by alveolar epithelial cells in situ is low but consistently detectable by immunohistochemistry (267). Considerably more Cx37 is expressed by bronchiolar epithelium, but this is still less than the level observed for pulmonary endothelial cells (267). To date, immunohistochemistry has not been done for Cx30.3 in the lung, although on the basis of levels of mRNA expression by isolated alveolar epithelial cells, we expect this to be fairly low as well.

In response to bleomycin-induced lung injury, the connexin profile is altered (2). In particular, the number of alveolar epithelial cells expressing Cx46 expression is significantly elevated. Cx43 is also upregulated at the protein level after lung injury, which may be due to alveolar epithelial hyperplasia (2, 140). Cx46-expressing alveolar epithelial cells do not express typical type II cell markers and thus may represent a distinct subtype of cells proliferating in response to injury (272). Interestingly, two connexins implicated in alveolar responses to injury, Cx26 and Cx46, have relatively limited permeability compared with Cx32 and Cx43 (40, 80, 204). Whether Cx26 is upregulated in response to lung injury in vivo remains to be determined. However, this leads to the appealing notion that upregulated Cx26 and/or Cx46 may, in part, help limit gap junctional communication to avoid propagating damage in response to injury. Limiting cell-cell coupling may also be related to increased alveolar epithelial cell growth, analogous to the studies described above. Consistent with this, we have found that human fetal type II cells are poorly coupled and show low levels of connexin expression (our unpublished observations).

Cx46, like most of the other connexins, is regulated at the level of mRNA expression, although connexin protein expression is often not directly proportional to levels of mRNA (115). As a further mechanism for regulation of gap junction channel composition used by alveolar epithelial cells, assembly of Cx46 into gap junctions is also regulated at the level of intracellular trafficking. In this case, type II cells retain Cx46 in the TGN, and type I cells, which also express Cx46, assemble it into gap junction channels (1, 59). Regulated connexin assembly is not unique to the lung, since regulated Cx46 trafficking has also been observed for osteoblastic cells (149). However, type I cells traffic Cx46 to the plasma membrane and form mixed Cx43/Cx46 gap junction channels (59). The mechanism of regulated Cx46 trafficking is not well understood at present; however, it is likely to be regulated by regulating Cx46 coassembly with Cx43 (59, 149). One intriguing possibility is that this change in connexin trafficking is related to changes in membrane trafficking that occur as cells differentiate from a type II cell phenotype (i.e., a surfactant-secreting cell) to a type I phenotype (which does not produce lamellar bodies), since this may reflect a reorganization of TGN-localized secretory pathways.

INTERCELLULAR COMMUNICATION PATHWAYS IN THE ALVEOLUS

On the basis primarily of dye transfer studies, it is clear that cultured monolayers of alveolar epithelial cells with a single phenotype communicate through gap junctions (1, 2, 115, 131, 132, 169). The extent of communication varies, but alveolar epithelial cells can at least communicate with nearest neighbors. The level of communication is also sensitive to cell phenotype,
consistent with the notion that gap junctional communication can be regulated by alveolar epithelial cells.

Primary alveolar epithelial cells cultured for 6 days in MEM plus 10% serum (day 6 cells) express connexins comparably with type I cells (e.g., high Cx43, no Cx26 or Cx32; see Table 1). Consistent with the notion that type I cells are only able to form Cx43 compatible gap junctions, day 6 cells form calcein- and LY-permeable gap junctions with HeLa cells transfected with Cx43 but not Cx32 (2). Note that day 6 alveolar epithelial cells also expressed Cx46, which is theoretically capable of forming heterotypic gap junction channels with Cx32 (293, 296). However, alveolar epithelial cells expressing both Cx43 and Cx46 did not form functional gap junction channels with HeLa/Cx32 cells, perhaps because cultured cells assemble most of the Cx46 into heteromeric complexes with Cx43 (59).

In the normal adult lung, heterocellular gap junctional communication between type II and type I cells is suggested by the studies of Ashino et al. (11), who demonstrated transmission of intracellular calcium transients initiated in type I cells to type II cells (described in CALCIUM TRANSIENTS AND HETEROCELLULAR REGULATION OF SURFACTANT SECRETION). As further evidence for heterocellular cell-cell communication between type II and type I cells, freshly isolated type II cells and type I cells cocultured with day 6 (or day 3) alveolar epithelial cells form functional gap junctions, as determined by dye transfer studies (115, 148).

Because type II cells express Cx26/Cx32 and Cx43, they have the capacity to form two independent classes of gap junction channels. However, type I cells express mostly Cx43 in the normal lung and thus may have only a single pathway for gap junctional communication. This implies that communication between type II and type I cells is typically mediated through Cx43-compatible gap junction channels. This also means that type II cells have a gap junction channel incompatible with their nearest neighbors in the uninjured lung (Fig. 4). In the injured lung, though, type II cell hyperplasia can increase the frequency of type II-type II cells in direct contact. Consistent with this, we observed Cx32-expressing type II cells in direct contact 7 days after instillation of bleomycin (2). Thus Cx32 may help provide type II cells with a “priority” channel for communication, independently of coupling pathways interlinking type II cells and type I cells. Theoretically, this has the potential to enable type II cells to simultaneously remain coupled to each other and functionally isolated from type I cells. To date, however, this has not been demonstrated, and whether there can be two functionally distinct zones of coupling in the alveolar epithelium remains an open hypothesis.

STRESSING OUT THE NEIGHBORS

Why alter gap junctional communication in response to injury? On the one hand, metabolic cooperation can help tissues share metabolites and enables a tissue to be more robust than a set of uncoupled cells would be (218). Metabolic cooperation is also good from the standpoint of helping cells resist moderate levels of injury by, say, enabling a cell to share glutathione pools with neighboring cells (49, 144, 200). Consistent with this, mild cytosolic stress (e.g., heat shock) or treatment with sodium arsenite increased gap junctional coupling by increasing connexin insertion into the ER and concomitant assembly into channels at the plasma membrane (279).

However, under high levels of stress, being interconnected can have the opposite effect; that is, either through metabolite depletion or through transmission of toxic substances, gap junctions might be more harmful than helpful. Some examples of this are outlined below. This also provides a rationale for isolating type II cells from type I cells during injury, since this has the potential to protect the type II stem cell population through decoupling. Whether this is the case, though, remains to be determined.

The transmission of cell toxicity through gap junction channels is sometimes referred to as the “bystander effect.” Experimentally, the bystander effect can be demonstrated by examining cocultures of cells expressing herpes simplex virus thymidine kinase (TK) coupled to TK-negative cells (10, 81, 87, 185). When treated with ganciclovir, both the TK-positive cells and normally resistant TK-negative cells are Fig. 4. Communication compartments in the alveolar epithelium. A: in the uninjured adult lung, alveolar epithelial cells are likely to be interconnected, to enable flow of metabolites between all cell-cell interfaces and calcium transients between type I and type II cells. B: a hypothetical model for changes in compartmentation in response to acute injury. Attenuation of Cx43-compatible gap junction channels by type II cells, either by gating or downregulation of Cx43 expression, could effectively isolate type II cells and create two distinct alveolar epithelial subcompartments. In this model, adjacent type II cells remain coupled by Cx32 gap junction channels that cannot connect to type I cells.
killed, due to TK-mediated phosphorylation of ganciclovir, which is then transmitted through gap junctions. Communication-deficient cells are resistant to the bystander effect. Consistent with gap junction-mediated transmission of toxic substances in a more native system, cell death induced by gamma irradiation is cell density dependent and is abrogated by gap junction inhibitors (194). Even radiation levels on the order of one alpha particle per five cells can create cytotoxic substances transmitted through Cx43 gap junction channels (312).

Cell-cell coupling can also play a role in cell death induced by exposure to oxidants. This is of relevance to the lung, because oxidant damage and protein nitrotyrosine formation are frequently associated with lung injury. For instance, patients suffering from ARDS and premature infants requiring ventilation show increased incidence of protein modification (102, 116, 253) as assessed by nitrotyrosine formation (12, 133). Bleomycin-induced lung injury shows a similar effect (261, 262, 305). Inhaled NO treatment for ARDS is also associated with protein nitrotyrosine formation (159). Tyrosine nitration is a modification that could interfere with tyrosine phosphorylation-mediated cell signaling pathways. Nitrotyrosine formation has also been linked with protein misfolding and aggregation, which underlie diseases linked to protein conformational defects, such as amyotrophic lateral sclerosis (94, 255). There are a few studies that suggest that peroxynitrite can inhibit gap junctional communication (25, 247) and function of tight junctions (130, 260). Whether this is due to a direct nitrotyrosine modification of connexins or whether this is downstream of a signaling cascade remains to be determined.

Interestingly, the toxicity of the peroxynitrite-generating compound 3-morpholinosydnonimine (SIN-1) toward alveolar epithelial cells is density dependent: cells at higher density are more sensitive to SIN-1 than cells plated at lower density (216), suggesting a possible role of gap junctional communication in peroxynitrite toxicity. This also suggests that any attenuation of gap junctional communication in response to SIN-1 may be an attempt to limit damage. Consistent with this possibility, type II alveolar epithelial cells are more resistant than type I cells to hydrogen peroxide (H₂O₂) injury (252). Although this correlates with the relative cell-cell coupling between these cell types (1, 169), type II cells have more catalase activity than type I cells, which will clearly have a protective effect (252). As another potential correlation, taurine protects from NO₂ lung injury and increases the number of gap junctions in the lung as assessed by morphometric analysis (105). Whether this is related to the level of coupling is less clear, though, since taurine has been associated with increased Cx32 activity in the liver (90) and has the opposite effect on reconstituted Cx32, where it is inhibitory (20).

In addition to causing cell toxicity, reactive oxygen species (ROS) can serve signaling functions (16, 190, 264, 300). ROS have been associated with hormone stimulation (207, 264) and specific gene activation (151, 187, 188) and can interact with signal transduction pathways (154, 223). Related to this point, glutathione has also been implicated in H₂O₂-mediated closure of gap junctions (129, 273, 308). H₂O₂-mediated closure of gap junctions also requires tyrosine kinase activity (126). This was mediated by Cx43 hyperphosphorylation (127), was inhibited by glutathione depletion (273, 308), and was not abrogated by free radical scavengers. Given these findings, it seems likely that the glutathione requirement observed for attenuating gap junctional communication may reflect a redox sensor activity as opposed to an antioxidant activity (190). Other signaling pathways can attenuate gap junctional communication and are associated with lung injury, such as MAP kinase cascades (37, 61, 249). Whether this is directly linked to changes in gap junctional communication between alveolar epithelial cells remains to be determined.

Also, whether hormones directly affect alveolar epithelial cell communication during lung injury is still an open question. For instance, increased transforming growth factor (TGF)-β is associated with both the acute and chronic phases of lung injury (139, 195, 217). TGF-β1 also can alter gap junctional communication; however, this effect is highly context dependent; there are studies showing that TGF-β1 can increase (50, 277), decrease (45, 235), or have no effect (168) on gap junctional communication, depending on cell type and incubation conditions. TGF-β1 has also been found to simultaneously upregulate Cx43 and suppress Cx37 expression by endothelial cells (165), which underscores the complexity of predicting cell responses to TGF-β stimulation, particularly when different TGF-β isoforms are taken into consideration.

In contrast, KGF treatment has also been shown to attenuate the extent of injury induced by bleomycin (66, 114, 307, 308), radiation (308), Pseudomonas infection (284), hyperventilation (209, 290), and hyperoxia (14). KGF also prevents glutathione depletion in the lung, which normally results from graft vs. host disease (314). Because KGF is a type II cell mitogen (36, 86, 192), it is tempting to speculate that part of the mitogenic and protective effects of KGF results from attenuating Cx43-mediated gap junctional communication and preserving the Cx32-mediated communication pathway specific for type II cells, as described in INTERCELLULAR COMMUNICATION PATHWAYS IN THE ALVEOLUS.

CONCLUSION

Alveolar epithelial cells show many different patterns of connexin expression. On the basis of the properties of connexins and the ways in which they interact with signaling pathways, we can infer similar functions for gap junctions in the alveolus to control cell growth and the intercellular distribution of metabolites and toxins. However, defining specific roles for gap junctions in alveolar epithelial function remains elusive. Even in the regulation of surfactant secretion, roles for gap junctional communication are obscured by potential cross talk with other intercellular signaling
mechanisms, such as putative paracrine ATP secretion. Perhaps cocultures of cultured cells will enable the interplay between these pathways to be elucidated, with the strong caveat that the cell phenotypes need to be rigorously defined with as many markers as possible. It seems likely that the next phase in understanding roles for gap junction proteins will also require transgenic mouse models. However, given the neonatal lethal phenotype of Cx26 (91), these studies may prove to be difficult and will require targeted deletion strategies. Defining heterocellular pathways to regulate surfactant secretion may also benefit from studies using P2Y₂ knockout mice (57), either separately or in conjunction with a type II cell-targeted connexin-deficient mouse. Cx32- and Cx46-deficient mice are viable but do not have an obvious pulmonary phenotype (103, 201). However, when stressed, these mice may then reveal specific functions for these connexins in that alveolar epithelium.

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