Mechanisms of Surfactant Exocytosis in Alveolar Type II Cells In Vitro and In Vivo

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Surfactant secretion must be regulated to maintain a low surface tension in the lung during various conditions such as exercise. In vitro studies reveal a slow, unique exocytotic process at the interface of stimulated and constitutive exocytosis. The exocytotic mechanisms and sites of regulation in vivo, however, are still poorly understood.

The alveolar epithelium is composed of cuboidal type II cells and squamous type I cells. Type II cells synthesize and secrete a surface-active agent (surfactant). Other functions, which will not be discussed here, are transport of solutes and alveolar repair following injury. Surfactant is a lipoprotein-like material that facilitates inspiration by lowering the surface tension at the air-liquid interface. This is achieved by secreted surfactant lipids positioned as a layer between air and fluid, preventing water molecules from exerting high molecular forces at the alveolar surface. The eminent role of surfactant in medicine first became clear in 1958, when it was shown that premature infants suffering from infant respiratory distress syndrome lack surfactant. The reduction of alveolar surface tension also counteracts lung edema formation by reducing the transmural pressure gradient between blood vessels and alveoli. Finally, surfactant supports alveolar host defense in many ways. Besides being a physical permeation barrier against some noxious material, some surfactant proteins have structural and functional properties similar to proteins of the complement system.

One of the most fascinating aspects of the surfactant system is the stabilization of a collection of ~300 million bubbles (alveoli), an inherently unstable structure, by a balanced reduction of the surface tension. In each respiratory unit (the acinus), surface tension must be adequately reduced to prevent its own collapse. This implies that there should be local regulatory mechanisms preventing depletion of the alveolar surface lipid film. A unique feature of natural (compared with industrial) surfactant is its compression dependence on the reduction in surface tension, such that during lung deflation, surface tension approaches 0 dyn/cm² (note that, according to LaPlace’s law, this is an important mechanism to keep small alveoli open). Along with this process, some squeezing out of material occurs. This material can be recycled, i.e., reincorporated into the lipid layer at the air-liquid interface, in two ways. First, it can be recycled within the respiratory acinus by recruitment from the subphase during inspiration. This recycling mechanism is a passive one and does not involve a cellular action. Second, part of “used” surfactant is recycled (via endocytosis) by the type II cell, resulting in a continuous surfactant turnover through this cell type. (Another route of surfactant recycling is through macrophages, which will not be discussed here.) The entire recycling process is slow; the turnover time for surfactant has been estimated at several hours (reviewed in Refs. 6 and 14). From estimates of flux, it was calculated that type II cells must be secreting between 14 and 47% of the lamellar body pool per hour (14). The turnover rate of surfactant is not constant. Consistently, secretion of surfactant by the type II cell is regulated, which has been demonstrated by numerous studies (see below). It is generally assumed that surfactant turnover through the type II cell is primarily governed by factors that control the rate of the exocytotic process of lamellar bodies (this assumption will be discussed below).

Physiologically, the most relevant condition associated with elevated surfactant turnover is probably exercise. It is well established that an increase in tidal volume, achieved by muscle force or mechanical ventilation, increases surfactant secretion. This effect may be considerable; 14-fold rates of secretion were reported in swimming rats (10).

What makes surfactant secretion so unique?

Lipid and protein components of surfactant are secreted by exocytosis of “lamellar bodies,” i.e., via fusion of surfactant-containing vesicles with the plasma membrane. Surfactant within lamellar bodies is densely packed as circular arrangements of lipid membranes. The exocytotic process of lamellar bodies bears some cytological, biochemical, and functional properties that distinguish it, in many aspects, from secretion in other cell types. First, lamellar bodies are very large (diameter ~ 1 μm), among the largest secretory vesicles in mammalian cells. Second, vesicle contents are secreted into a small fluid volume termed hypophase, subphase, or alveolar lining fluid (ALF), which covers the epithelial cell layer and separates it from the air space. Its average thickness was estimated to be 200 nm, varying from a few nanometers to almost micrometer sizes (2). Third, the secreted product (surfactant) is lipophilic and therefore poorly soluble in an aqueous environment. This probably results in the accumulation of various macromolecular aggregates of surfactant in the ALF. Notably, circular structures of surfactant in the ALF suggest that, in some cases, vesicle contents from lamellar bodies are secreted into the ALF without significant transformation. The mechanisms of surface lipid film formation are not entirely understood.
A challenge to match demand for surfactant with supply

As noted above, the type II cell must react with enhanced secretion of surfactant before the alveolar lining becomes depleted of it, and under physiological conditions this is most relevant during exercise. Does this mean that there is no signal to enhance surfactant secretion unless the surface tension increases? Certainly not, because a single deep breath is already sufficient to enhance phospholipid release in the rat lung. Notably, volume and not volume per time is the signal, because ventilation by increased frequency at constant tidal volume does not elicit this response. These experiments suggested that lung distention rather than systemic changes accompanying hyperventilation (PCO₂, PO₂, pH) augments secretion. An important piece of evidence in favor of the idea that inspiration causes stretch of the type II cell was added recently by demonstrating significant increases of the surface area of the epithelial basement membrane (reflecting epithelial cell deformations) in response to a physiological extent of lung inflation (>42% total lung capacity) (12). The effect is not mediated through the autonomous nervous system, because the loss of lamellar bodies in response to large breaths is not affected by bilateral vagotomy (9). Direct evidence that stretch of type II cells by itself is able and sufficient to trigger secretion was provided by experiments on isolated type II cells grown on Silastic membranes, which responded to a single stretch by intracellular Ca²⁺ mobilization and phospholipid release (13).

Therefore, it is reasonable to assume that mechanical stretch of the type II cell during an enhanced inspiration (“sigh”) is a direct stimulus for secretion. Stretch-mediated enhancement of surfactant secretion during exercise prevents a loss of alveolar surfactant. There is a rapid increase of the total amount of phospholipid in the alveolar compartment after onset of swimming in rats, consistent with an increased alveolar surfactant pool during exercise, which recycles from and to the air-liquid interface at an elevated rate. This intra-alveolar surfactant pool is long-lived, since it was sustained for at least 2 h following termination of swimming (10).

Large-breath-induced enhancement of surfactant secretion is accompanied by a dramatic loss (~30%) of lamellar bodies from type II cells within 1 h (9). What this clearly indicates is that lamellar body synthesis does not keep pace with lamellar body loss, at least during initial periods of stimulation. Does this imply that at some point during exercise, the supply of lamellar bodies to the apical plasma membrane becomes rate-limiting for secretion (an idea that would challenge the concept of regulated secretion)? How many lamellar bodies would a Tour de France bicyclist loose on his 6-h ride through the French Alps? Each of his type II cells contains ~150 lamellar bodies, a smaller number than in most other secretory cell types. Considering that a single deep breath is a stimulus for secretion, a regulated exocytic process comparable to that in neuroendocrine cells would quickly deplete his cellular vesicle pool, assuming that each stimulus would cause the fusion of at least one vesicle (this is a minimum assumption for regulated exocytosis, in which readily releasable vesicles instantaneously fuse in response to a Ca²⁺ signal). The low number of vesicles but the high frequency of stimulation during strenuous exercise is an apparent problem. Here we summarize currently available data on the mechanisms and regulation of surfactant secretion and discuss how a type II cell could keep the balance between stimulated vesicle fusion and smooth, long-term release of surfactant.

Surfactant secretion can be stimulated by various intracellular signaling pathways

Various in vivo, ex vivo, and in vitro techniques have been employed in the past to study the regulation of surfactant secretion: lung lavage, lung compliance, ultrastructural studies, isolated perfused lung, lung slice techniques, and others. But it was the development of cell isolation methods in the 1980s that enabled the study of signaling cascades and the mechanisms and pathways of stimulation in purified type II cell preparations. An in-depth review of the multitude of studies dealing with this issue would greatly exceed the scope of this article (the interested reader is referred to Ref. 8). In short, the activation of multiple intracellular messenger systems can independently stimulate secretion, including cAMP-protein kinase A, phospholester-protein kinase C (PKC), and elevation of the cytoplasmic Ca²⁺ concentration ([Ca²⁺]). In addition, pharmacological evidence suggested an involvement of calmodulin in stimulated surfactant secretion. It was also shown that the effects of phorbol esters or activators of cAMP are additive to those of Ca²⁺ ionophores.

It is still largely unknown which pathways are activated in a defined in vivo condition associated with increased surfactant secretion and how these pathways are activated in a physiological setting. As noted above, since a single stretch activates...
Regulated secretion of surfactant in vitro is a slow and multiphasic process

In general, exocytosis proceeds in distinct steps (7), the most crucial being vesicle fusion with the plasma membrane. The prefusion phase comprises all steps until fusion competence of a secretory vesicle is achieved. This includes vesicle transport to the plasma membrane, “docking” with the plasma membrane, and a series of reactions denoted “priming” (depending on the system studied, priming reactions may take place before or after docking). The subsequent postfusion phase is related to content dispersal into the extracellular space, which depends on various factors, including the geometry and properties of the fusion pore and the solubility and dispersibility of vesicle contents.

A standard technique to study the regulation of surfactant secretion is to measure the accumulation of radiolabeled phospholipids in cell supernatants of isolated alveolar type II cells in primary culture. It is evident that, in these types of experiments, the bath solution is only a vague substitute for the poorly defined ALF, and mechanical forces may be absent. Only very recently have techniques been developed that enable the study of single type II cell function within the isolated lung (1). Naturally, the time course of lipid accumulation in the bath solution is a function of all exocytotic steps, yielding neither information about the site of regulation nor about the rate-limiting steps within the overall process. These in vitro studies revealed that, irrespective of the mode of activation, the appearance of phospholipids in the cell supernatant is a very slow process, which terminates up to several hours after the instance of stimulation (Fig. 1). Hence, a single stimulus can cause a sustained secretory process in vitro.

Does this mean that fusion of vesicles with the plasma membrane continues for hours following a single stimulus? Most likely not. According to fluorescence techniques enabling the detection of single fusion events as recently developed (5), the rate of fusion events is much faster than the rate of phospholipid accumulation (Fig. 1). In general, fusion activity is highest within the first 5 min and levels off thereafter, terminating within a maximum period of ~30 min. The difference in time course between vesicle fusion with the plasma membrane and surfactant release into the extracellular space is demonstrated in Fig. 1.

The implication of these findings is that surfactant secretion consists of at least two phases, a prefusion and a postfusion phase, which are clearly separated in time. Which cellular mechanisms underlie these phases and which are regulated?

The postfusion phase: fusion pores or related structures limit release in vitro

Long-lasting purse string-like structures of the plasma membrane at the site of single vesicle fusion can be observed with confocal laser scanning microscopy in type II cells (Fig. 2). The dwell time of surfactant within a fused lamellar body is usually very long, up to several hours, demonstrating that the postfusion phase is rate-limiting for secretion, at least in isolated cells (3). It should be noted, however, that these apparent invaginations at the site of fusion as visualized with high-resolution microscopy (Fig. 2) may have little in common with initial fusion pores as measured with electrophysiological (capacitance) techniques (7). They might represent complex macromolecular structures associated with the cytoskeleton, which suggested by their large diameters of up to 1 μm or more compared with a few nanometers of initial fusion pores (3). It tempting to speculate that contractile elements in conjunction with these pores might regulate the size of the pore and therefore the rate of surfactant release through it. In line with this notion, we found by fluorescence recovery of photobleaching that the rate of diffusion of FM 1-43 (compare with Fig. 2) through a pore was increased by an elevation of Ca2+ (unpublished observations). This points to a possible physiological role of Ca2+ as regulator of the postfusion phase of surfactant secretion. Finally, it should be kept in mind that the presence and mechanical properties (distensibility) of each cell type, and these properties have not yet been sufficiently determined. It could also be possible that, in vivo, stretch induces ATP release from lung cells, causing secretion via activation of purinergic receptors and phosphoinositide hydrolysis, resulting in both Ca2+ mobilization and the activation of PKC. Extracellular ATP is one of the most potent stimuli of surfactant secretion in vitro (8), but essentially nothing is known about its origin and role in the alveolus.

**FIGURE 2.** Release of surfactant through a horizontal section of a fusion pore in a single living type II cell in primary culture. Laser scanning microscopy of surfactant (left) and of the plasma membrane plus surfactant (right) are shown. A, B, and C are images from the same cell before vesicle fusion (A), 20 min after vesicle fusion (B), and 40 min after vesicle fusion (C). Note the slow "protrusion" of surfactant.
The prefusion phase: the interface of constitutive and stimulated exocytosis

Since there is only one macromolecular complex (surfactant) in a fused vesicle, Fick's law does not apply to describe its permeation through the fusion pore. Nevertheless, we can postulate that the amount of secretion per time \((S/t)\) be proportional to the average area of single fusion pores \((r^2 \pi)\), assuming circular fusion pores and the number of fusion pores \((n)\)

\[ S/t = F r^2 \pi \]

where \(F\) is a yet-undefined factor related to viscoelastic properties of surfactant.

A type II cell monolayer in primary culture always exhibits a certain number of fusions even without stimulation. These are defined as constitutive fusion events. If there is also some amount of constitutive fusion in vivo (which is likely), surfactant delivery to the air-liquid interface could theoretically be efficiently enhanced by increasing the radius of these fusion pores, even without a change in the fusion rate. This effect, if present, does not appear to be sufficient, however, to satisfy the demand of surfactant during stimulation.

Type II cells have developed a fusion machinery allowing them to temporarily increase the fusion rate by manyfold without depleting their stored vesicles within short periods of time. First, type II cells do not exhibit a pool of vesicles in a docked, readily releasable state, at least in vitro (3a, 4, 5). This implies that, unlike in the classically defined condition of regulated exocytosis, type II cells do not respond to a \(Ca^{2+}\) signal (elicited, for instance, by the flash photolysis of caged \(Ca^{2+}\)) with immediate and synchronous fusions of many vesicles, but rather with cumulative fusion events starting with highly variable delays of many seconds to minutes (3a). In primary cell cultures, the number of stimulated fusion events per cell is in the range between 0 and 10. The likelihood of fusion in response to a short \(Ca^{2+}\) signal is highest ~1 min thereafter. The range between 0 and ~10. The likelihood of fusion in...
Regardless of whether the response to stretch is mediated by Ca\textsuperscript{2+} mobilization alone or by purinergic stimulation in an autocrine fashion, Ca\textsuperscript{2+} is definitely the most important second messenger involved in either condition. This is demonstrated by a clear Ca\textsuperscript{2+} dependence of agonist-induced fusion and, moreover, by a close temporal correlation between fusion activity and the course of the Ca\textsuperscript{2+} signal. ATP-induced Ca\textsuperscript{2+} elevations are longer than ultraviolet-flash-induced Ca\textsuperscript{2+} signals and are the result of both intracellular Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} entry (3a). Both phases influence the magnitude and temporal behavior of the fusion response. Accordingly, the amount of ATP-induced fusion is dependent on the magnitude of the integrated Ca\textsuperscript{2+} signal (3a). Naturally, as noted, there may be a common downstream messenger in all Ca\textsuperscript{2+}-mediated signals, such as calmodulin. The efficacy of this Ca\textsuperscript{2+} sensor could be modified by various factors, explaining why repetitive signals may be subject to either tachyphylaxis or potentiation. A candidate for potentiation is PKC, which has been shown to sensitize Ca\textsuperscript{2+}-induced fusion by modulating the exocytotic machinery in other cell types and which is also a strong activator of surfactant secretion in type II cells. GTP-binding proteins may also play a role, because GTP\textsubscript{i}S induces surfactant secretion under “Ca\textsuperscript{2+}-clamped” conditions.

On the basis of in vitro data (3a), we propose a model for vesicle processing and fusion that is consistent with slow (compared with neuroendocrine cell types), continuous secretion and that appears to be perfectly adapted to avoid quick depletion of the cellular vesicle pool (Fig. 3). We postulate a more-or-less random distribution of vesicles at various stages, such that some but not all cells happen to have vesicles close to fusion (Fig. 3). Since there is a clear relationship between the duration and amplitude (integral) of a Ca\textsuperscript{2+} signal and the amount of fusion, it is thought that, depending on the shape of the Ca\textsuperscript{2+} signal, vesicles are processed to some degree toward fusion competence in each cell. In case of long (or repetitively spiking) Ca\textsuperscript{2+} signals, many vesicles from various initial stages may undergo fusion (Fig. 3). On a mechanistic basis, the simplest explanation would be that lamellar bodies are constantly “pushed” toward the apical membrane by mechanical forces. Cytoskeletal structures, however, may act as both barrier and motor by preventing the contact of the vesicle membrane with the plasma membrane. Each Ca\textsuperscript{2+} signal could activate gelosin and thereby solate the cortical actomyosin. This idea is strongly supported by the finding that actin depolymerization enhances fusion and that major stimuli of surfactant secretion cause a reduction of F-actin (11). Once a close apposition between membranes is reached, elevated local Ca\textsuperscript{2+} concentrations would complete the process of fusion. It should be noted here that in vitro fusion assays for isolated lamellar body membranes are still lacking. Therefore, we do not yet know the Ca\textsuperscript{2+} dependence of the membrane fusion event itself. Cytosplasmic Ca\textsuperscript{2+} measurements cannot exclude local Ca\textsuperscript{2+} elevations at the site of fusion, even if they are not detected with common Ca\textsuperscript{2+} indicators.

The exocytotic process described above is unique in mamalian cells. It would probably be the result of an adaptation to long-lasting stimuli such as exercise, and it is clearly at the interface of the classic definitions of constitutive and stimulated fusion. In such a scenario, Ca\textsuperscript{2+}-induced fusion is no longer the only rate-limiting step for secretion. Ca\textsuperscript{2+} would instead exert a “permissive role” for secretion, the rate being determined by additional factors, such as lamellar body formation and transport.

**Future perspectives**

Most of what we know on a cellular and a molecular level about surfactant secretion comes from studies on isolated type II cells in vitro. In comparison, little is yet known about the mechanisms of secretion in vivo, in particular about intracellular vesicle pools, their dynamic behavior, and the postfusion phase of release. If we want to gain more insight into these issues, we shall have to refine new methods and techniques and apply them to the intact alveolus. The first steps in this direction have already been made (1).

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**References**