Review

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Lamellar Body Exocytosis by Cell Stretch or Purinergic Stimulation: Possible Physiological Roles, Messengers and Mechanisms

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Key Words

Exocytosis • Fusion • Hemifusion • Surfactant • Alveolus • Lung • Type II cell • Stretch • Mechanotransduction • ATP

Abstract

A major function of the pulmonary alveolar type II cell is the secretion of surfactant, a lipoprotein-like substance, via exocytosis of secretory vesicles termed lamellar bodies (LBs). The process of surfactant secretion is remarkable in several aspects, considering stimulus-delayed fusion activity, poor solubility of vesicle contents, long hemifusion lifetimes, slow fusion pore expansion and active, actin-driven content release. Cell stretch as well as $P2Y_2$ receptor stimulation by extracellular ATP are considered the most potent stimuli for LB exocytosis. For both stimuli, elevation of the cytoplasmic Ca^{2+} concentration $[Ca^{2+}]_c$ is a key step. This review summarizes possible physiological roles and pathways of stretch- or ATP-induced surfactant secretion and discusses molecular mechanisms controlling the pre-, hemi- and postfusion phase, in comparison with neuroendocrine release mechanisms.

Introduction

Surfactant, short for "surface active agent", is a lipid-rich, lipoprotein-like substance, secreted into the lumen of the pulmonary alveolus. It consists of mainly phospholipids and four specific surfactant proteins (SP) [1, 2]. Surfactant is a crucial secretory product during and after birth, its continuous and accurate secretion and function is indispensable throughout life. The discovery of surface tension as the major component of retractive forces in the lung was made as early as 1929 [3], but it was not until the 1950s and early 1960s that active surface material from the lung was isolated and characterized [4, 5]. Its deficiency causes infant respiratory distress syndrome (IRDS) [6]. The lamellar body (LB) within the type II pneumocyte was identified as the intracellular vesicular storage site of surfactant [7]. The LB is a large organelle (about 1-2 μm Ø) that stores surfactant in an extremely compact, lamellar conformation (recently reviewed in [8]). Although the number of type II pneumocytes is about twice the number of type I pneumocytes, they represent only about 7% of the alveolar surface [9]. The first convincing evidence in favor of an exocytotic surfactant release mechanism was

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provided by electron microscopy (EM) studies [10]. The phospholipid composition of LBs isolated from type II pneumocytes is similar to that of whole lung surfactant obtained from broncho-alveolar lavage (BAL) [11, 12]. Essentially all alveolar surfactant phospholipids are secreted via exocytosis of LBs [13]. In addition to the phospholipids, all four surfactant proteins (SP-A, SP-B, SP-C, and SP-D) which account for about 10% by weight, can be obtained by BAL [8, 14]. Their respective distributions within LBs and BAL are different: The small hydrophobic SP-B and SP-C are localized within LBs and co-secreted with all other LB contents. Both proteins are believed to play an important role in squeezing out non-dipalmitoyl phosphatidylcholine (DPPC) components during film formation and compression at the air-liquid-interface, which results in a highly DPPC-enriched surface film [8, 15]. In contrast, the large hydrophilic SP-A and SP-D are secreted largely independently of LB contents by other routes. Although SP-A has an inhibitory effect on LB exocytosis [16], these proteins appear to be mainly involved in pulmonary host defense. Surfactant secretion has been extensively reviewed elsewhere [17-21]. This review focuses on the regulation of the exocytotic process of LBs by the two probably most potent and physiologically important stimuli: alveolar stretch or extracellular ATP. In particular, we shall discuss and distinguish between mechanisms before and after fusion of the LB with the plasma membrane, in comparison with neuroendocrine release mechanisms.

Ventilation and mechanical effects on alveolar epithelial cell shape

It has been known for decades that large lung inflations stimulate surfactant secretion [22-27]. When the lung is distended during inspiration, at least two different forms of mechanical stress (defined as force per unit of area) can act on the epithelium of the respiratory zone:

1. Re-opening of collapsed airways can induce shear stress on the epithelium and connective tissue. This shear stress may play an important role for ventilator-induced lung injury during artificial ventilation of diseased lungs (reviewed in detail in [28]).

2. Probably even more relevant is the fact that alveoli are subject to a tensile strain (= stretch, defined as a change in length in relation to the initial length). Under the assumption of the alveolus as a homogenously inflated balloon, each spot should be stretched in an equi-biaxial way, i.e. by the same length in all directions. However, this strain model is likely a gross simplification, due to the uneven alveolar shape and an alveolar wall that is probably not homogenous in its elastic properties [29], resulting in an unisotropic stress distribution [30, 31]. In fact, it is still a matter of debate how much inflation is actually needed to stretch individual epithelial cells after unfolding of connective tissue (analogous to the extension of an accordion). From alveolar surface to volume relationships, Gil and Weibel concluded that alveolar surface changes are primarily due to folding and unfolding of septa before plastic tissue changes occur [32]. On the other hand, a morphometric analysis by Tschumperlin and Margulies revealed significant changes of the epithelial basement membrane surface area above 40% of total lung capacity [33], indicating epithelial cell deformation at physiologically relevant lung volumes.

Stretch-induced signalling of alveolar type II cells

Live cell imaging of single cells during static or cyclic stretch is a methodological challenge, and the elucidation of stretch-induced Ca^{2+} signals with high temporal and special resolution is just beginning to emerge [34]. Alveolar type II cells in culture (mostly isolated from rat lungs) are very sensitive to tensile strain and respond to a single stretch - increasing the cell surface area only by about 10 % - with an elevation of the cytoplasmic Ca^{2+} concentration $[Ca^{2+}]_c$ and stimulated surfactant secretion [35, 36]. Thus, it is likely, that the increase of surfactant secretion, which is observed after only a single inflation of the isolated perfused lung [25], is the result of a related epithelial cell stretch. Recently however, the notion of the type II pneumocyte as the alveolar stretch sensor was challenged by an *in situ* study, based on fluorimetric measurements of $[Ca^{2+}]_c$ [37], suggesting that type I pneumocytes are the primary responders to alveolar inflation. Using gap junction blockers, Ashino and collegues concluded that Ca^{2+} signals can promote from type I cells to type II cells, consistent with the notion that small signalling molecules can permeate gap junctions [38]. A distinct but related mechanism was recently proposed using mechanically stretched co-cultures of type I and type II rat pneumocytes: These data suggest that alveolar type I cells act as mechanosensors, releasing ATP in response to stretch activation, which subsequently stimulates type II cells in a paracrine way [39].

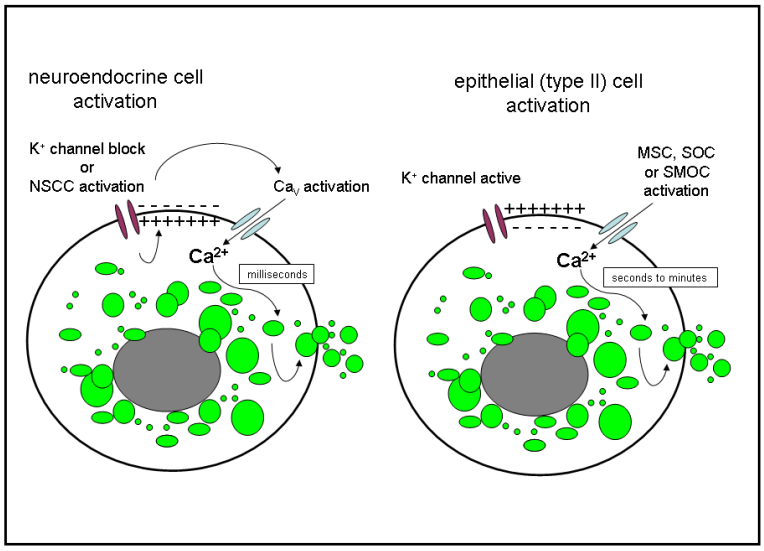

Fig. 1. Stimulus-secretion-coupling in neuroendocrine cells (left) and epithelial (type II) cells (right). In neuroendocrine cells, a depolarization of the membrane potential (e.g. due to K^+ channel block or cation channel activation) triggers the activation of voltage-dependent Ca^{2+} channels (Ca_V), which leads to Ca^{2+} entry and subsequent fusion of secretory vesicles (green) with the plasma membrane within milliseconds. In type II epithelial cells, the resting membrane potential is part of the electrochemical force, which drives Ca^{2+} ion influx presumably through one or several of the following channel types: mechanosensitive channels (MSC), store-operated channels (SOC) or second messenger operated channels (SMOC). Vesicle fusion with the plasma membrane is triggered within seconds to minutes. In both cell types, intracellular stores also contribute to the increase in cytoplasmic Ca^{2+} concentration (not shown).

All these models, however, converge in an elevation of $[Ca^{2+}]_c$ in the type II pneumocyte, where surfactant is stored and released by LBs. Ca^{2+} entry from the extracellular space and Ca^{2+} release from intracellular stores both contribute to this $[Ca^{2+}]_c$ elevation [35, 36]. Ca^{2+} entry from the extracellular space is indeed essential for surfactant release from stretched type II cells, which renders mechano-sensitive ion channels as particularly promising candidates [35]. Nevertheless, the molecular basis for the stretch-induced Ca^{2+} entry - in particular the molecular entities of stretch-induced Ca^{2+} channels - are still obscure. It should be noted that depolarizing membrane potentials do not result in Ca^{2+} entry into type II pneumocytes, because those cells do not express functional voltage-dependent Ca^{2+} channels (Ca_V) [40]. Thus, the entry of extracellular Ca^{2+} through Ca_V is not possible, which is a fundamental difference to neuroendocrine secretion, where Ca_V is an important mediator in excitation-secretion-coupling. Consequently, in contrast to neuroendocrine secretion, non-selective cation channels can only be considered to contribute to the rise of $[Ca^{2+}]_c$ if they are permeable for Ca^{2+} [41], since indirect activation via voltage-gated Ca^{2+} entry is absent. This difference between epithelial and neuroendocrine secretion is illustrated in Fig. 1.

Among Ca^{2+} permeable channels which might be involved in stretch-induced Ca^{2+} entry in the type II cell, three different functional categories come into consideration (Fig. 1):

Mechanosensitive channels (MSC), store-operated channels (SOC), and second messenger-operated channels (SMOC).

1. Mechanosensitive channels (MSC): Recently, several Ca^{2+} permissive members of the transient receptor potential (TRP) channel family have been proposed as stretch-sensitive channels; and channels of the epithelial Na^+ channel (ENaC)/degenerin family are other candidates [42-49]. Tissue specific mRNA expression analysis (RT-PCR and in situ hybridization) suggest several potentially mechanosensitive TRP channels of the V and C subfamilies in the lung as candidates, but functional evidence is yet lacking [50].

2. Store-operated channels (SOC): Since cell stretch in the type II cell causes Ca^{2+} release from thapsigargin-sensitive intracellular stores [35, 36], the activation of a store-operated Ca^{2+} entry pathway must be taken into consideration. Thapsigargin is an inhibitor of the endoplasmic Ca^{2+} ATPase and frequently used as a pharmacological tool to specifically stimulate SOC in the absence of phosphoinositide breakdown [51]. Although different conductances and ion selectivities of SOC have been presented , a highly Ca^{2+} selective channel termed CRAC (Ca^{2+} release activated Ca^{2+}) channel is best characterized [52]. CRAC channel activation involves physical migration of endoplasmic reticulum (ER)-resident STIM proteins to ER–plasma membrane junctions and subsequent aggregation of the Ca^{2+} influx channel Orai (recently reviewed by [53]). To this end, the existence and potential physiological role of CRAC channels in type II cells remains to be elucidated.

3. Second messenger-operated channels (SMOC). The impact of static or cyclic stretch on extracellular matrix

(ECM)/integrin signalling, gene expression, cytokine release and type II cell function/phenotype has been reviewed in detail elsewhere [22, 27, 54-57]. Hindering integrin interaction with the ECM by RGD (Arg-Gly-Asp) peptides inhibits the stretch-induced Ca^{2+} signal (G. Fois, unpublished observation). Recently, it was shown that variable stretch patterns influence surfactant secretion [58]. These observations leave space for speculations that mechanically induced signalling molecules might affect LB exocytosis, however possible links between focal adhesions and Ca^{2+} permeable channels are yet unclear.

Possible causes of ATP release in the alveolus

As noted, stretch during a deep lung inflation is considered the most important if not the only physiologically relevant stimulus of surfactant secretion [23-25, 27, 59-64], and stretch-induced cellular ATP release could represent a key element of pulmonary alveolar mechanotransduction. An autocrine mechanism of purinergic (ATP) stimulation was proposed after hypotonic swelling in a surrogate cell line of type II pneumocytes (A549 cells) [65], but it is still unclear if osmotic cell volume changes occur in alveolar cells *in vivo* at all. The intriguing question whether or not ATP is indeed involved in stretch-induced surfactant secretion in the native lung remains unclear. In 1983, Gilfillan et al. [66] presented first evidence for extracellular ATP as a stimulator of surfactant secretion using a perfused rat lung slice preparation. Their work was inspired by the discovery of purinergic nerves [67], which utilize ATP as a neuro(co)transmitter and innervate several visceral organs including the lung, terminating at sites close to alveolar type II cells [68]. Although a clear physiological role of ATP release from pulmonary purinergic nerves has never been established, ATP emerged as the most potent physiological agonist of surfactant secretion, despite its mysterious origin and presence under various conditions of stimulation. One possible route of ATP release is through anion channels (reviewed in detail [69]). However, although various anion channels have been identified in fetal and adult pneumocytes [70], their involvement in alveolar ATP release has not yet been demonstrated. It is unlikely that membrane stress alone is the reason for ATP release from cells under physiological conditions, because moderate amounts of stretch are in general tolerated without signs of membrane damage [35, 36, 71, 72]. However, ATP may exit all cells under conditions of cell damage, and this could be the case in hyperinflation-induced lung injury. Indeed, Gajic et al. found signs of reversible cell membrane stress failure in rats ventilated with high tidal volumes using propidium iodide as a cell-impermeable marker of cell damage [72]. In addition, we found ultrastructural evidence for membrane damage in type II pneumocytes stretched on silastic membranes [71]. In summary, in pathophysiological conditions, ATP leakage from wounded cells could influence type II cells and surfactant secretion in an autocrine/paracrine way.

Interestingly, new findings suggest that purinergic (ATP) control of surfactant secretion could be stimulated by bacterial lipopolysaccharides (LPS), presumably via upregulation of purinergic receptors of the $P2Y_2$ subtype (see below), resulting in an increased Ca^{2+} signal and a pronounced exocytotic response of LBs to treatment with ATP [73]. Hence, sensitation of the purinergic system during inflammation might augment physiological stimuli.

Purinergic receptors and their signalling cascades in type II pneumocytes

Since ATP is a very potent stimulus of surfactant secretion [74-77], the purinergic signalling pathway has been intensively investigated and is detailed in several excellent reviews [1, 17, 18, 21, 78-80]. ATP and UTP are equally potent to stimulate surfactant secretion [81], indicating signalling via a $P2Y_2$ receptor (formally termed P_{2U} receptor), consistent with pharmacological data [74]. In addition, surfactant secretion is also stimulated by adenosine [75], and all its receptor subtypes (A_1, A_{2A}, A_{2B} and A_3) have been identified in isolated type II cells [82].

The ATP-induced Ca^{2+} signal in type II cells consists of at least 2 phases, the "peak" and the "plateau" phase, analogous to a multitude of other cell types [83]. The "peak" is independent of extracellular Ca^{2+} and hence a result of intracellular Ca^{2+} release from stores, presumably the endoplasmic reticulum [83]. The "plateau" depends on extracellular Ca^{2+} and is most likely a result of Ca^{2+} entry through the plasma membrane [83]. The "plateau" is not as stable as in many other cell types but frequently superimposed by transient Ca^{2+} elevations of yet unknown origin that coincide with single LB fusions with the plasma membrane [84]. Ion channels which are responsible for the ATP-induced Ca^{2+} influx and "plateau" phase in type II pneumocytes are yet unknown, nor is their mechanism of activation. In analogy to stretch-

induced Ca^{2+} entry (see above), SOCs and/or SMOCs may account for ATP-induced Ca^{2+} entry (Fig. 1). Since type II cells are subject to considerable changes of shape (and possibly cell volume) after stimulation with ATP (unpublished observation), not even MSCs can be excluded in this context.

Type II cell stimulation with ATP is coupled to both adenylate cyclase (AC; cAMP/PKA) and phospholipase C (PLC; IP3/DAG/PKC) signalling [85, 86]. cAMP is also generated due to stimulation of $ß_2$-adrenergic receptors with isoproterenol [87], which may be important during labor [88]. Accordingly, cell-permeable cAMP analogues themselves stimulate surfactant secretion [89]. The mechanism by which cAMP elicits this exocytotic response is not yet known, but its potency is much lower than that of an elevation of the cytoplasmic Ca^{2+} concentration or phorbol esthers (Haller and Dietl, unpublished observation), which also stimulate secretion (see below).

Activation of PLC results in phosphoinositide (PIP_2) breakdown and generation of inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) [74]. The PLC isoform PLC-β3 was identified by RT-PCR and immunoblotting as the only isoform present in type II cells [82]. DAG generation in type II cells in response to extracellular ATP is biphasic: The first peak occurs immediately (about 10 s) after ATP treatment and coincides with IP_3 formation as a result of PLC activation [74]. The second DAG peak follows with a delay of 10 - 15 min and coincides with formation of phosphatidic acid (PA) or - in the presence of ethanol - phosphatidylethanol, indicating the coactivation of a phosphatidylcholine-specific phospholipase D (PLD) [74, 81, 90]. mRNA for both PLD isoforms, PLD1 and PLD2, have been identified by RT-PCR in type II cells [80].

ATP-induced DAG activates protein kinase C (PKC), and again, several PKC-isoforms have been identified by RT-PCR in type II cells [82, 91, 92]. DAG, released from a caged compound by UV-photolysis, or cell-permeable phorbol esthers (like TPA, OAG) that directly activate PKC, are themselves strong stimulators of surfactant secretion [83, 93], further supporting that PKC is involved in downstream stimulation of surfactant secretion via extracellular ATP. In fact, phorbol esthers degranulate type II cells quite effectively without a concomitant elevation of the cytoplasmic Ca^{2+} concentration, but intracellular Ca^{2+} chelation abolishes this effect [83]. It was therefore proposed that PKC sensitizes the exocytotic machinery for the action of Ca^{2+} [83, 94], possibly by phosphorylation of SNAP-25 as demonstrated in an insulin-secreting cell line [95].

In this context it is reasonable to assume that IP_3 formation accounts for ATP-induced Ca^{2+} mobilization from intracellular stores [96, 97]. However, the effects of IP_3 were never directly investigated in type II pneumocytes. It is not clear if Ca^{2+} stores mobilized by ATP are different from or overlap with Ca^{2+} stores mobilized by stretch.

The process of surfactant exocytosis

The described orchestrated activity of bioactive compounds generated by stretch or purinergic stimulation makes a single mechanism or molecular target of exocytosis quite unlikely. Nevertheless, the elevation of $[Ca^{2+}]_c$ appears to be a central common component by which LB exocytosis from alveolar type II pneumocytes is triggered. This assumption is based on the following findings:

1. A variety of Ca^{2+}-dependent agonists and Ca^{2+} ionophores stimulates surfactant secretion, most specifically flash photolysis of caged Ca^{2+} [83, 98-100].

2. Intracellular Ca^{2+} chelation blocks LB exocytosis stimulated by different agonists [83, 100].

3. There is an almost linear correlation between the integrated $[Ca^{2+}]_c$ over time and the amount of surfactant secretion after stimulation with ATP [83].

Since exocytosis is an extremely conserved mechanism in evolution, the predominant role of Ca^{2+} in this cell type is not surprising. However, in contrast to synaptic or neuroendocrine secretion, where secretory vesicle fusion occurs within < one millisecond up to > hundred milliseconds following the rise of $[Ca^{2+}]_c$ [101, 102], the kinetics and time scale of surfactant release are delayed by several orders of magnitude: The delay between the ATP-induced Ca^{2+} signal and LB fusion with the plasma membrane is between seconds and several minutes, and the delay between LB fusion and surfactant release is of the same order of magnitude, occasionally even longer (hours) [84, 96, 99]. The reasons for this discrepancy between type II cell and neuroendocrine secretion are probably manifold (see also Fig. 1), but mostly related to structural features: First, there is neither morphological nor functional evidence in favour of so-called “docked” vesicles in type II cells. It rather appears that a few LBs are bulging out the plasma membrane, resulting in tongue-like folds of the plasma membrane after fusion [10]. Second, as noted above, epithelial cells lack voltage-gated Ca^{2+} channels and thus have no “active zones”, as present in presynaptic terminals.

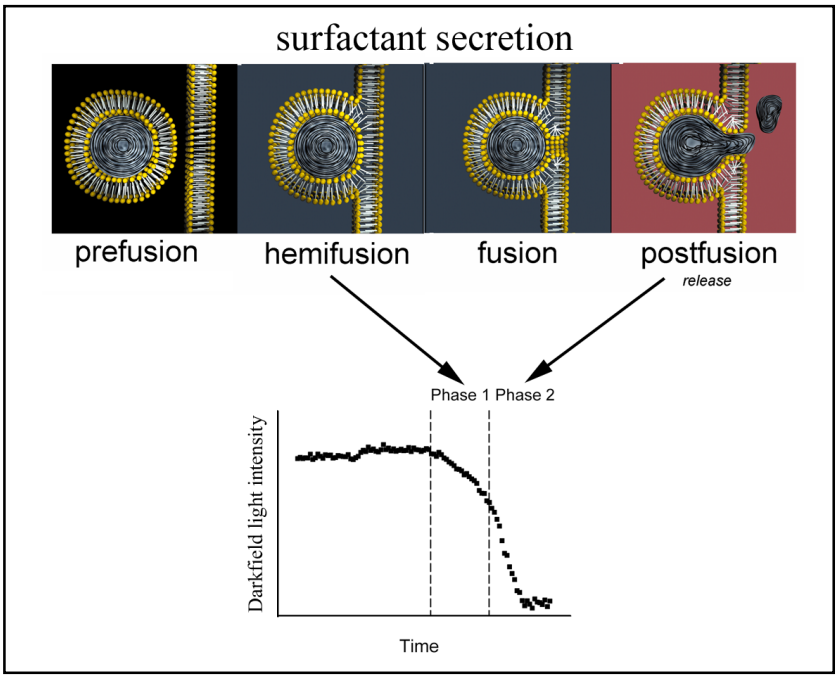

Fig. 2. Top: Schematic illustration of the three phases of surfactant secretion from type II pneumocytes, indicated by different background colors. Prefusion, hemifusion and postfusion. The hemifusion and postfusion phases are separated by the instance of fusion pore formation (fusion). Bottom: Original tracing of the darkfield light intensity measured from a single LB within a living type II cell. Arrows indicate that phase 1 of the darkfield scattered light intensity decrease (SLID) corresponds to the presumed hemifusion phase, and phase 2 to the postfusion phase. Modified from Ref [106]. For details see text.

As summarised in Fig. 2, the exocytotic process of surfactant could be divided into three distinct steps, which are differently controlled. Each one can be rate-limiting for the secretion of surfactant:

1. The prefusion phase: This denotes all events that make the LB fusion-competent, including trafficking and docking of the LB to the plasma membrane, creating a morphological and biochemical contiguity that enables lipid merger and fusion pore formation.

2. The hemifusion phase and fusion: The exocytotic hemifusion state ("fusion-through-hemifusion" theory) is a postulate (reviewed in detail by [103]) which denotes the merger of the outer leaflet of the limiting vesicle membrane with the inner leaflet of the plasma membrane. Experimental evidence for hemifusion is scarce and indirect [104, 105], however our own observations by darkfield microscopy provide evidence in favour of hemifusion intermediates in the type II cell, with a duration from not measurable up to about 9s [106].

Fusion pore formation takes place when the vesicle membrane and plasma membrane become a lipid bilayer continuum, and thus the vesicle lumen and the extracellular space an aqueous continuum. These steps have been intensively investigated in biological and artificial systems and are hallmarks of exocytosis [107].

3. The postfusion phase: This denotes fusion pore expansion and release of LB contents. In the type II cell, fusion of a LB with the plasma membrane (synonymous with fusion pore formation) can precede surfactant release (secretion) for long periods (up to hours under cell culture conditions, see also below).

Control of the prefusion phase

LB fusion with the plasma membrane is a stereotypic response to an elevation of $[Ca^{2+}]_c$, triggered eg. by UV flash photolysis of caged Ca^{2+} [99], the Ca^{2+} ionophore ionomycin [100, 108-110], cell stretch [35, 36], or purinergic stimulation (as discussed above). The threshold $[Ca^{2+}]_c$ for LB fusion is about 320 nM and thus far lower than in cells with small vesicles (up to hundreds of µM) [99]. In contrast to far more intensively investigated neurotransmitter release [107], the molecular mechanisms by which Ca^{2+} elicits LB fusion are still less clear. SNARE (soluble *N*-ethylmaleimide-sensitive-factor attachment receptor) proteins and the presumed Ca^{2+} sensor synaptotagmin are major candidates for LB fusion , and synaptotagmin II knockout mice have indeed impaired stimulated mucin secretion from airway epithelial cells, which shares many features with surfactant secretion [112, 113]. Yet, no information is available on surfactant secretion in these mice.

Another discussed Ca^{2+} sensor that might be responsible for LB fusion is synexin (annexin VII), a GTP hydrolyzing and phospholipid binding protein, displaying fusogenic activity in various cell types [114]. Several studies suggest involvement of synexin in Ca^{2+}-dependent LB

fusion with the plasma membrane [115-119]. This is consistent with the finding that the non-hydrolyzeable GTP analogue GTPγS stimulates LB fusion with the plasma membrane without stimulating LB-LB fusion [120].

Another way by which Ca^{2+} could mediate LB fusion with the plasma membrane is the disassembly of cortical actin, which is located beneath the plasma membrane. The idea that exocytotic fusion is limited by the cortical F-actin network, which represents a physical barrier, preventing vesicle contact with the plasma membrane, was proposed as a general principle for exocytosis in non-excitable cells [121]. The notion that exocytotic fusion is enabled whenever the fusion hindrance by cortical actin is removed gains support by recent fusion studies on proteoliposomes and synaptic vesicles, showing that the SNARE complex syntaxin 1, SNAP-25, and synaptobrevin can act as a constitutively active fusion machine, independently of synaptotagmin and Ca^{2+} [122]. Evidence that disassembly of the actin network does in fact enhance exocytotic LB fusion comes from a study where basal surfactant secretion was augmented after decreasing cellular F-actin levels with botulinum C2 toxin [123]. Beside being a fusion clamp that inhibits fusion, actin may promote exocytosis by various mechanisms at a pre- or postfusion stage (see below). Possible roles for actin in the exocytotic process have recently been summarized in an excellent review by Malacombe et al. [124]. A possible Ca^{2+} sensor for this task is scinderin, a Ca^{2+}-dependent actin filament-severing protein, which binds phosphatidylserine, PIP_2 and actin [124]. A role for scinderin was demonstrated in mucous cells [125], but information in type II cells is yet lacking.

Control of the hemifusion phase

As a general rule, hemifusion is favored by lipids that support negative curvature of the membrane leaflets (facilitating stalk formation), such as fatty acids, whereas fusion pore formation and expansion is favored by positive curvature lipids, such as lysophospholipids [126]. It was proposed that vesicles transiently hemifused with the presynaptic membrane enable the extremely fast kinetics of neurotransmitter release (< 100 µs after the rise of $[Ca^{2+}]_c$) [127], and this is consistent with EM and FRAP investigations revealing docked vesicles in a hemifused state [104, 105]. Owing to the long delay (several seconds) between the elevation of $[Ca^{2+}]_c$ and fusion pore formation in LBs [99], it is very unlikely that LBs reside in a "docked", hemifused state. Nevertheless, using a newly developed hemifusion assay based on darkfield scattered light intensity decrease (Fig. 2), we have recently shown that hemifusion intermediates in type II pneumocytes can last for up to about 9 seconds [106]. It is yet unclear if LB hemifusion intermediates are always destined to proceed to fusion, or if they can be transient and revert to a prefused state. In any case they represent a significant stage of surfactant exocytosis that is potentially subject to regulation by SNARE proteins or lipid metabolites.

Among the discussed bioactive lipid metabolites generated by purinergic stimulation that might promote LB fusion, PA is a particularly promising candidate because it induces negative plasma membrane curvature due to its small polar head group in combination with two fatty-acyl side chains [128], possibly promoting lipid merger with the vesicle membrane and hemi-fusion [129]. Although direct evidence for this mechanism has never been presented in type II cells, it is supported by the observation that some type II cells exhibit a biphasic fusion activity (immediately after ATP treatment and > 10 min later), where the early response coincides with Ca^{2+} mobilization, whereas the delayed one occurs with a delay reported for PA generation [84].

Mechanisms to control surfactant release in the postfusion phase

Our knowledge about the release mechanism of surfactant from native type II cells in the lung still mainly comes from electron microscopy (EM) investigations, where tubular myelin, a highly ordered array of membranes found by transmission EM in pulmonary alveoli, was postulated as the extracellular conversion product of released LBs [130, 131]. Some images display fusion pores of various diameters (up to > 1 µm), through which surfactant appears to be squeezed (example in Ref. [80]). However, a dynamic resolution of this process within the intact lung is still beyond the limits of current imaging techniques.

Nevertheless, advanced microscopy techniques do allow the visualization of the entire exocytotic process – including fusion pore formation, fusion pore expansion and surfactant release in either primary cultured type II pneumocytes [84, 96, 132, 133] or *in situ* (isolated perfused lung), however yet with limited resolution [37]. Using fluorescence microscopy in isolated type II cells, tubular myelin cannot be clearly identified, and it

appears that the hydrophobic, poorly soluble complex of surfactant must be actively squeezed through a reluctantly opening fusion pore. Several experimental data are in line with this notion:

1. Fusion pores in type II cells have the tendency to expand slowly [84]. The reason for this slow expansion is not yet clear but may well be related to cytoskeletal elements. An elevation of $[Ca^{2+}]_c$ accelerates pore expansion [84].

2. Fusion pores in type II cells can act as mechanical barriers for release. This was concluded from experiments using laser tweezers as force generators [134].

3. An actin coat must be formed around the fused LB before surfactant can be squeezed out, probably by contraction of the actin coat [133]. These observations using GFP-actin-transfected pneumocytes were consistent with earlier observations that actin reorganization accompanies LB exocytosis [135, 136].

Prior to LB contraction by the actin coat, the fused LB transiently swells, presumably by fluid uptake through the fusion pore [106]. The physiological significance of postfusion LB swelling is yet unknown, although this may represent a mechanical load to the limiting LB membrane and surrounding structures. Postfusion vesicle swelling was observed in numerous secretory cell types [137]. Swelling of the vesicle and hydration of vesicle matrix might be important for fusion pore stabilization and expansion in mast cells [138].

Intracellular Ca^{2+} immobilization with the chelator BAPTA-AM, as well as the PLD inhibitor C2-ceramide hindered ATP-induced actin coat formation in type II cells [133]. In the absence of an actin coat, however, cells were unable to secrete surfactant, and fused LBs remained in this position ("wait" position) as filled bags connected with the plasma membrane [133]. This suggests that Ca^{2+} and PLD activation (in response to ATP) may control the postfusion (release) phase of surfactant exocytosis.

Is actin-powered release the reason for fast surfactant secretion *in vivo*?

There is ample evidence that surfactant secretion by pharmacological stimulation or stretch *in vivo* is fast and occurs within min (reviewed in detail in [21, 139]). Isolated type II pneumocytes *in vitro*, however, exhibit a considerable variability in the time course of surfactant release from single LBs, ranging from minutes to hours [84]. Recent experiments using GFP-actin-transfected pneumocytes suggest that a slow time course of release relates with the inability to produce an actin coat around fused LBs [133]. Hence, it may be that the fast surfactant secretion observed *in vivo* is due to a more effective actin coating as compared to isolated cells.

Outlook

The slow process of stimulated surfactant exocytosis renders the type II pneumocyte an ideal cell model to investigate time-resolved actions of physiologically relevant modulators of exocytosis.

Currently, one of the most intriguing questions is how actin coat formation is limited to the fused LB without affecting all LBs in a cell stimulated with ATP. It appears that there are spacially and/or temporarily confined processes that cannot spread throughout the cell. Yu and Bement recently disclosed a signalling pathway in oocytes involving a PLD-dependent DAG incorporation into the granule membrane after fusion, triggering Cdc42/N-WASP-induced actin assembly [140]. Although this still has to be investigated in type II cells, PLD would be expected to primarily control the post-fusion phase, because neither alcohol, which inhibits PLD-induced DAG generation, nor the PLD inhibitor C2 ceramide, blocked LB fusion activity [83, 133]. As outlined above, a potential way by which lipid metabolites might control surfactant exocytosis is the promotion of hemifusion as well as the expansion of the fusion pore.

Inducible, type II pneumocyte-specific knockout animals of the $P2Y_2$ receptor would probably be of great help to distinguish stretch- from ATP-dependent signalling pathways and to clarify potential autocrine (ATP release) mechanisms involved in stretch-induced surfactant secretion.

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