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ARTICLE

Michaela Ross · Silke Krol
Andreas Janshoff · Hans-Joachim Galla

Kinetics of phospholipid insertion into monolayers containing the lung surfactant proteins SP-B or SP-C

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Abstract The lung surfactant proteins SP-B and SP-C are pivotal for fast and reversible lipid insertion at the air/liquid interface, a prerequisite for functional lung activity. We used a model system consisting of a preformed monolayer at the air/liquid interface supplemented with surfactant protein SP-B or SP-C and unilamellar vesicles injected into the subphase of a film balance. The content of SP-B or SP-C was similar to that found in lung lavage. In order to elucidate distinct steps of lipid insertion, we measured the time-dependent pressure increase as a function of the initial surface pressure, the temperature and the phosphatidylglycerol content by means of surface tension measurements and scanning force microscopy (SFM). The results of the film balance study are indicative of a two-step mechanism in which initial adsorption of vesicles to the protein-containing monolayer is followed by rupture and integration of lipid material. Furthermore, we found that vesicle adsorption on a preformed monolayer supplemented with SP-B or SP-C is strongly enhanced by negatively charged lipids as provided by DPPG and the presence of Ca^{2+} ions in the subphase. Hence, long-range electrostatic interactions are thought to play an important role in attracting vesicles to the surface, being the initial step in replenishment of lipid material. While insertion into the monolayer is independent of the type of protein SP-B or SP-C, initial adsorption is faster in the presence of SP-B than SP-C. We propose that the preferential interaction between SP-B and negatively charged DPPG leads to accumulation of negative charges in particular regions, causing strong adhesion between DPPG-containing vesicles and the monolayer mediated by Ca^{2+} ions, which eventually causes flattening and rupture of attached liposomes as observed by in situ SFM.

M. Ross · S. Krol · A. Janshoff · H.-J. Galla (✉)
Institut für Biochemie, Westfälische Wilhelms-Universität,
Wilhelm-Klemm-Strasse 2, 48149 Münster, Germany
E-mail: gallah@uni-muenster.de
Tel.: +49-251-8333200
Fax: +49-251-8333206

Keywords Insertion kinetics · Scanning force microscopy · Film balance · Surfactant protein B · Surfactant protein C

Abbreviations *DPPC*: 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine · *DPPG*: 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol · *EDTA*: ethylenediaminetetraacetic acid · *Hepes*: *N*-2-(hydroxyethyl)piperazine-*N*′-2-ethansulfonic acid · *LB*: Langmuir-Blodgett · *SP-A*: surfactant protein A · *SP-B*: surfactant protein B · *SP-C*: surfactant protein C · *SP-D*: surfactant protein D · *SFM*: scanning force microscopy

Introduction

The pulmonary surfactant is a complex mixture of lipids and surfactant-associated proteins secreted by the alveolar type II cells into the alveolar space of the lung. It forms tightly packed monolayers at the alveolar interface, which are capable of lowering the surface tension and thus preventing a collapse of the alveoli. Analysis of lung lavage has revealed that pulmonary surfactant mainly consists of 85–90% lipids, especially dipalmitoylphosphatidylcholine (DPPC) and phosphatidylglycerols (PGs) besides other mainly unsaturated phospholipids, fatty acids, cholesterol and proteins (Possmayer et al. 1984). Furthermore, four surfactant-specific proteins with different structural properties have been identified: hydrophilic surfactant proteins A (SP-A) and D (SP-D), and surfactant proteins B (SP-B) and C (SP-C) which are both hydrophobic. SP-A is a glycoprotein, assembled from 18 polypeptide chains, which binds phospholipids and carbohydrates. It is required for the formation of tubular myelin, a lattice-like membrane structure that is thought to be the precursor of the monolayer (for review, see McCormack 1998). SP-D is composed of four subunits, each of which has three identical disulfide-linked glycosylated polypeptides of about 43 kDa. It may play a role in the host-defense system of the lung (for reviews, see Crouch 1998; Reid

1998). SP-B is a cysteine-linked homodimer, each monomer being composed of 79 amino acids, exhibiting a molecular weight of 8.7 kDa. It induces bilayer contact sites and subsequent lipid mixing between bilayers (Oosterlaken-Dijksterhuis et al. 1992; for review, see Hawgood et al. 1998). SP-C is one of the most hydrophobic natural peptides, owing to a high content of Val, Ile and Leu. It is a monomer with 35 amino acid residues and exhibits a molecular weight of 4.2 kDa. SP-C adopts an α-helical conformation and has two palmitoyl groups covalently linked to the polypeptide chain (for review, see Johansson 1998). It was shown by Oosterlaken-Dijksterhuis et al. (1991a, 1991b) that SP-C and SP-B in phospholipid vesicles or a monolayer at the air/water interface enhance the adsorption of phospholipids on the air/water interface.

To function effectively, a surfactant requires two apparently contradictory attributes. First, the surfactant needs to be capable of lowering the surface tension to very low values during expiration. Second, replenishment of surfactant at the air/liquid interface must be fast upon expansion in order to maintain the low surface tension. For instance, DPPC lowers the surface tension to near zero values at compression but it functions poorly since it adsorbs and spreads only slowly on the air/water interface (Goerke and Clements 1986; Pastrana-Rios et al. 1994, 1995). Since DPPC still exists in the ordered (gel) state at 37 °C it is able to withstand high surface pressures, but, on the other hand, displays slow spreading kinetics. In contrast, unsaturated phospholipid components of the surfactant are known to spread more rapidly at the air/water interface due to their low phase transition temperature, but monolayers collapse already at surface pressures well below those required in vivo. Along with the unsaturated phospholipids, the hydrophobic surfactant proteins SP-B and SP-C are thought to be responsible for rapid formation of surfactant monolayers at the air/water interface and the insertion and removal of phospholipids during the expansion and compression of the surfactant film (for review, see Pérez-Gil and Keough 1998). The mechanism by which SP-B and SP-C enhance the phospholipid adsorption and subsequent insertion at the air/water interface is still a matter of discussion.

This paper deals with the spreading kinetics of protein-free vesicles composed of DPPC and DPPG to SP-B- and SP-C-containing monolayers. By using in situ scanning force microscopy (SFM) we achieved production of snapshots of the different states of the insertion process, revealing a two-step mechanism in which adsorption and vesicle fusion are the two main steps.

Materials and methods

Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) were purchased from Avanti Polar Lipids (Alabaster, Ala., USA) and used without further purification. Chloroform and methanol were HPLC grade and were purchased from Sigma-Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany), respectively. The buffer solution used to hydrate lipid films consisted of *N*-2-(hydroxyethyl)piperazine-*N*′-2-ethansulfonic acid (Hepes) from Merck (Darmstadt, Germany) supplemented with ethylenediaminetetraacetic acid (EDTA) from Fluka (Neu-Ulm, Germany). The preparation of vesicles was carried out with a Liposofast-miniextruder from Avestin (Ottawa, Canada). The porcine SP-B was a grateful gift from Dr. Creuwels, former member of the work group of Prof. Dr. L. van Golde at the University of Utrecht, Netherlands; human recombinant SP-C was a generous gift from Byk-Gulden Pharmaceuticals (Konstanz, Germany). The amino acid sequence is GIPCCPVHLKRLLIVVVVVVLIVVVIVGALLMGL and the two cysteine residues are palmitoylated.

Vesicle preparation

Phospholipid mixtures (DPPC/DPPG with molar ratios of 4:1, 1:1 or 0:1) were dissolved in chloroform/methanol (1:1, v/v) and dried under a stream of nitrogen at 50 °C. Remaining solvent was removed for at least 3 h at 50 °C in a vacuum oven. The lipid films were hydrated by adding a buffer containing 25 mM Hepes and 0.1 mM EDTA. The suspension (5 mM) was kept for 10 min at 50 °C in a water bath and was vortexed for 30 s. Then, it was stored for 10 min at 4 °C and incubated for another 10 min at 50 °C. The procedure of heating, vortexing and keeping at 4 °C was repeated twice. The resulting multilamellar vesicles (MLV) were converted into small unilamellar vesicles (SUV) at 50 °C by membrane extrusion using a polycarbonate membrane with a pore diameter of 50 nm.

Kinetic studies

The experiments were performed using a preformed monolayer composed of DPPC/DPPG in a molar ratio of 4:1, representing the ratio of neutral and negatively charged lipids found in bovine lung lavage (Possmayer et al. 1984). In the case of protein/lipid films, a protein content of 0.2 mol% for SP-B and 0.4 mol% for SP-C was chosen in accordance with earlier investigations (Amrein et al. 1997; von Nahmen et al. 1997a, 1997b; Galla et al. 1998; Krol et al. 2000a, 2000b) and with respect to the fact that in lung lavages the content of hydrophobic proteins was found to be less than 1% (Curstedt et al. 1987; Hall et al. 1992). The monolayer was spread at the air/water interface of a Wilhelmy film balance with a 25 mL Teflon trough (15.4 cm × 2.5 cm). The subphase consisted of 25 mM Hepes (pH 7.0) and 3 mM calcium chloride and was stirred continuously by a magnetic bar. The lipid/protein film was compressed with a computer-controlled barrier to a defined surface pressure between 15 mN/m and 45 mN/m. After a constant pressure had been maintained for 10 min, vesicle suspensions were injected through an injection hole into the subphase with a Hamilton syringe. The final lipid concentration in the subphase was 20 μmol/L. In order to measure the temperature dependence of the protein-mediated insertion process, vesicles with DPPC/DPPG (molar ratio 4:1) were used. To determine the influence of negatively charged phospholipids on the insertion of material from the subphase, lipid composition in the vesicles was varied (0, 20 and 50 mol% DPPG).

Insertion of lipids from the subphase was studied at 20 °C and 37 °C by either following the surface pressure with time at constant total area or the change of total area at constant surface pressure. In the latter case a quantitative determination of the insertion rate was possible. As soon as lipid vesicles were inserted into the monolayer the computer-controlled barriers moved apart in order to maintain a constant surface pressure. The increase in area with time was linear at the beginning and decreased after a certain time because of the loss of lipid material in the subphase in the course of lipid insertion. Alternatively, relaxation of the

monolayer could also account for the observed effect. The initial slope was determined and divided by the initial area in order to obtain an insertion rate independent of the area (unit: s^{-1}). An example of a corresponding area/time diagram is shown in Fig. 1b. In order to illustrate the insertion of phospholipids into preformed monolayers, only pressure/time diagrams and diagrams comparing different insertion rates are presented subsequently (e.g. Fig. 1a).

Langmuir-Blodgett transfer

In order to prepare Langmuir-Blodgett (LB) films the Wilhelmy balance used for kinetic studies was equipped with a dipping device. Mica plates were freshly cleaved and dipped into a pure water subphase before spreading DPPC from chloroform/methanol solutions (1:1, v/v). After 10 min the monolayer was compressed to a pressure of 45 mN/m with an initial rate of 1.79 cm^2/min and equilibrated for 30 min. The DPPC film was then deposited onto the mica sheet at a speed of 0.7 mm/min, while maintaining constant pressure. Prior to the transfer of the second monolayer to the DPPC-coated mica plate, the DPPC monolayer on the mica was dried for at least 1 h, rendering the surface hydrophobic.

The second monolayer was also transferred by vertical LB dipping. Prior to film transfer the protein-containing monolayer was compressed to the desired pressure of 30 mN/m and vesicles were added to the subphase. The DPPC-precoated mica plate was dipped into the subphase through the protein-containing monolayer with attached vesicles at a speed of 0.7 mm/s. The speed of the barriers only adjust the material loss of a transferred monolayer, and in this course the surface pressure was allowed to increase up to 50 mN/m during the transfer. So different stages of the insertion process should be observable with SFM. Once in the subphase, the supported bilayer is mounted in an SFM fluid-cell without being exposed to air. In order to interrupt the vesicle binding and vesicle insertion process, after the transfer the buffer was replaced by pure water to remove excess Ca^{2+} ions.

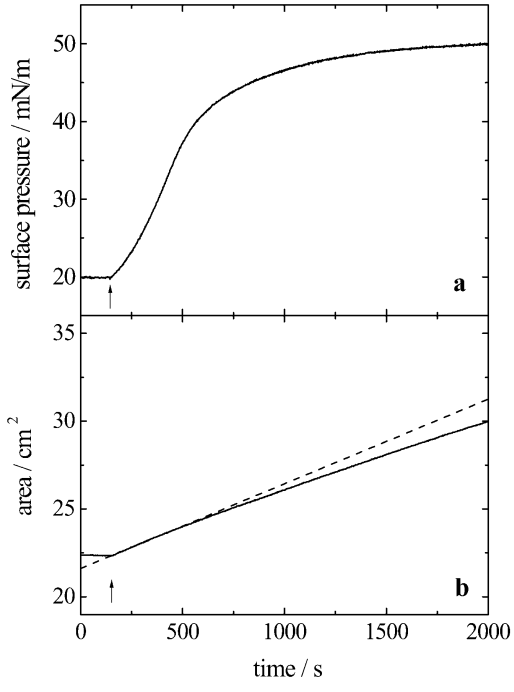


Fig. 1 Surface pressure versus time curve (**a**) and area versus time curve (**b**) of SP-B (0.2 mol% of the dimeric form) containing DPPC/DPPG (4:1) monolayers at 20 °C. Exemplary analysis of the insertion rate of curve **b** by linear fitting the initial slope (*dashed line*). The *arrows* indicate vesicle injection into the subphase

Scanning force microscopy

Surface images of the LB films were obtained using a Nanoscope IIIa Bioscope microscope from Digital Instruments (Santa Barbara, Calif., USA) operating in contact mode. Contact probes with a nominal spring constant of 0.06 N/m (NP Digital Instruments) were used. All measurements were performed in water.

Results

The effect of temperature

The surface pressure versus time curves obtained at 37 °C are presented in Fig. 2. Both SP-C- (Fig. 2a) and SP-B-containing (Fig. 2b) monolayers showed a sigmoidal increase in surface pressure after injecting the vesicle suspensions. The time required to reach the constant maximum surface pressure of 50 mN/m was about 4 min and identical for both proteins at initial surface pressures of 35 mN/m and 45 mN/m. Only at 30 mN/m did the vesicle insertion into a SP-B-containing monolayer take over 25 min, whereas the process was already finished after only 10 min if SP-C was used. The evaluation of area versus time diagrams revealed almost identical insertion rates for films containing either SP-B or SP-C at initial surface pressures of 30 mN/m (Fig. 2c). The long lag-time found for the SP-B-containing film at 30 mN/m might be due to the fact that monolayers had to be compressed to an initial surface pressure above 30 mN/m in order to observe vesicle insertion in the relevant time scale (data not shown). The surface pressure of 30 mN/m seems to represent the threshold value of vesicle insertion at 37 °C. However, with increasing surface pressure, the initial insertion rates of vesicles into the SP-B-containing monolayer rose faster than the insertion into a monolayer containing SP-C.

Further investigations were performed at 20 °C in order to study the influence of temperature on the insertion kinetics. Unlike at 37 °C, insertion of phospholipid vesicles at 20 °C occurred at an initial surface pressure as low as 15 mN/m, revealing different pressure/time curves for SP-B- and SP-C-containing films (Fig. 3). In the case of SP-C-containing monolayers (Fig. 3a), the insertion of vesicles was characterized by a sigmoidal increase of surface pressure following a long linear increase of surface pressure up to 25–30 mN/m and a steep slope at 30–50 mN/m. In the case of monolayers containing 0.2 mol% SP-B (Fig. 3b), a rapid sigmoidal increase of surface pressure up to 40 mN/m was observed directly after injecting a suspension of phospholipids. Interestingly, for all measurements the rate of vesicle insertion decreased at a surface pressure of 40 mN/m and the final surface pressure of 50 mN/m was reached after at least 30 min. The evaluation of the corresponding area versus time curves is presented in Fig. 3c. In the presence of SP-C the insertion rate increased continuously with the initial surface pressure, whereas for SP-B a maximal insertion

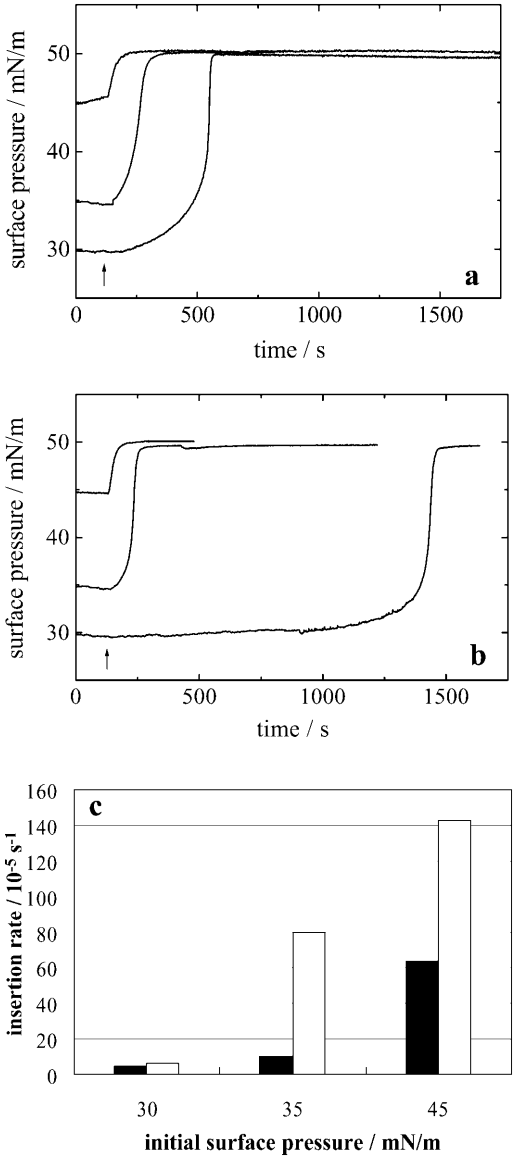


Fig. 2 Time-dependent pressure increase of **a** SP-C (0.4 mol%) and **b** SP-B (0.2 mol% of the dimeric form) containing DPPC/DPPG (4:1) monolayers induced by vesicle (DPPC/DPPG 4:1) injection (*arrow*) under the preformed monolayer. Experiments were performed at 37 °C on a subphase containing 25 mM Hepes and 3 mM $CaCl_2$, pH 7.0. **c** Insertion rates of vesicles added to SP-C (*black bars*) and SP-B (*white bars*) containing monolayers at 37 °C as a function of initial surface pressure obtained from analysis of corresponding area/time curves

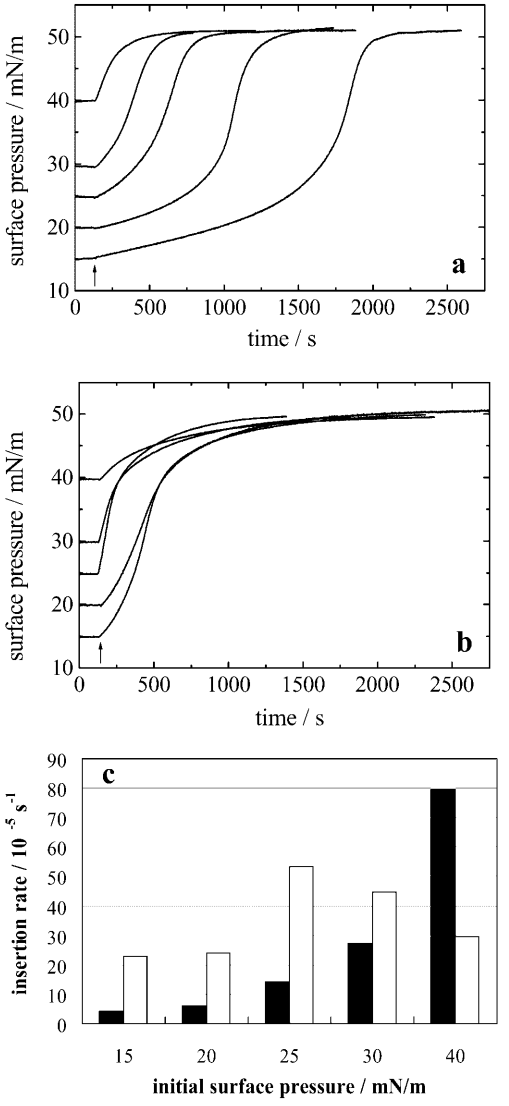


Fig. 3 Time-dependent pressure increase of **a** SP-C (0.4 mol%) and **b** SP-B (0.2 mol% of the dimeric form) containing DPPC/DPPG (4:1) monolayers upon vesicle (DPPC/DPPG 4:1) injection (*arrow*) in the subphase. Experiments were performed at 20 °C on a subphase containing 25 mM Hepes and 3 mM $CaCl_2$, pH 7.0. **c** Insertion rates for SP-C (*black bars*) and SP-B (*white bars*) containing monolayers at 20 °C as a function of initial surface pressure obtained from analysis of corresponding area/time curves

rate at an initial surface pressure of 25 mN/m was found. The initial insertion rate of vesicles in the presence of SP-B was at least three times faster than in the case of SP-C. Only at an initial surface pressure of 40 mN/m did SP-C show faster vesicle insertion kinetics.

Influence of DPPG content in liposomes

In order to study the influence of negatively charged phospholipids at 20 °C, DPPC vesicles with 0, 20 and 50 mol% DPPG were injected into the subphase at an initial surface pressure of 20 mN/m (Fig. 4). In the presence of SP-C, for pure DPPC vesicles, a slow, almost linear increase in surface pressure was observed up to a value of 25 mN/m, followed by a decreasing insertion rate (Fig. 4a). When 20 mol% DPPG were present in the vesicles, the increase in surface pressure was identical to neat DPPC up to a value of 25 mN/m and then diverged markedly with a considerable increase in insertion rate. The insertion of lipids from vesicles containing 50 mol% DPPG showed a steep linear increase in surface pressure, leveling off at 45 mN/m. Essentially the same results were obtained from SP-B-containing

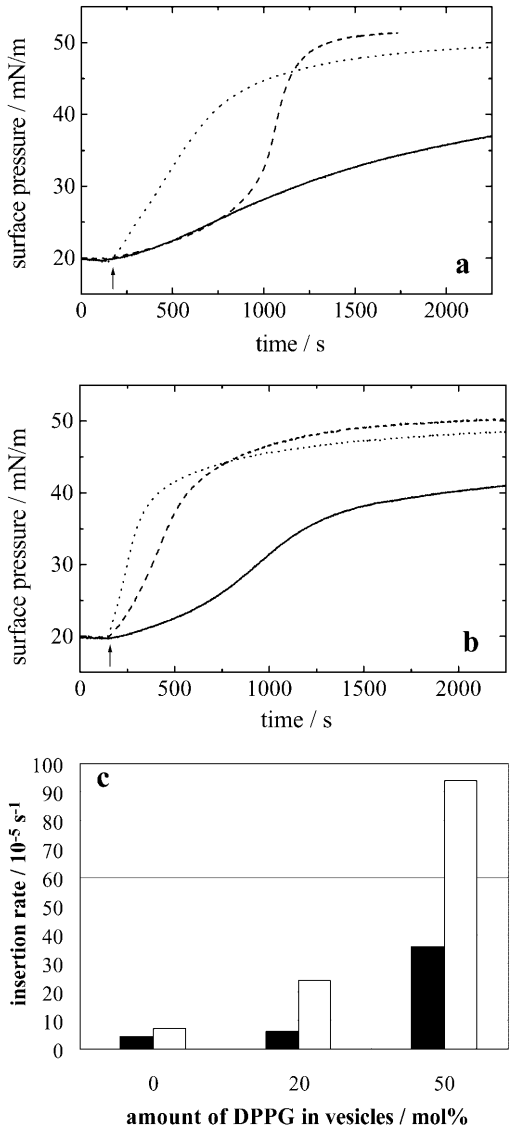


Fig. 4 Time-dependent pressure increase of **a** SP-C (0.4 mol%) and **b** SP-B (0.2 mol% of the dimeric form) containing DPPC/DPPG (4:1) monolayers induced by vesicle injection (*arrow*). The amount of DPPG in the vesicles was varied: *solid line*, 0 mol%; *dashed line*, 20 mol%; *dotted line*, 50 mol%. Experiments were performed at 20 °C on a subphase containing 25 mM Hepes and 3 mM $CaCl_2$, pH 7.0. **c** Insertion rates for SP-C (*black bars*) and SP-B (*white bars*) containing monolayers at 20 °C as a function of the DPPG amount present in the vesicles obtained from analysis of corresponding area/time curves

monolayers, although the influence of DPPG was more even pronounced than in the presence of SP-C. However, SP-B-containing monolayers clearly distinguish between vesicles containing 20 mol% DPPG and those of neat DPPC, which is in sharp contrast to the experiments with SP-C that showed no significant difference (Fig. 4b). SP-B-containing monolayers display faster insertion in the presence of vesicles with 20 mol% DPPG than in the absence of anionic lipids. A content of 50 mol% DPPG in the vesicle suspensions caused a steep increase in surface pressure in the case of SP-B which was about twice as fast as in the case of a monolayer containing SP-C (Fig. 4c). It is noteworthy that, in the presence of calcium, negatively charged lipids are not absolutely necessary for successful lipid insertion but considerably enhance the insertion rate, especially in the presence of SP-B.

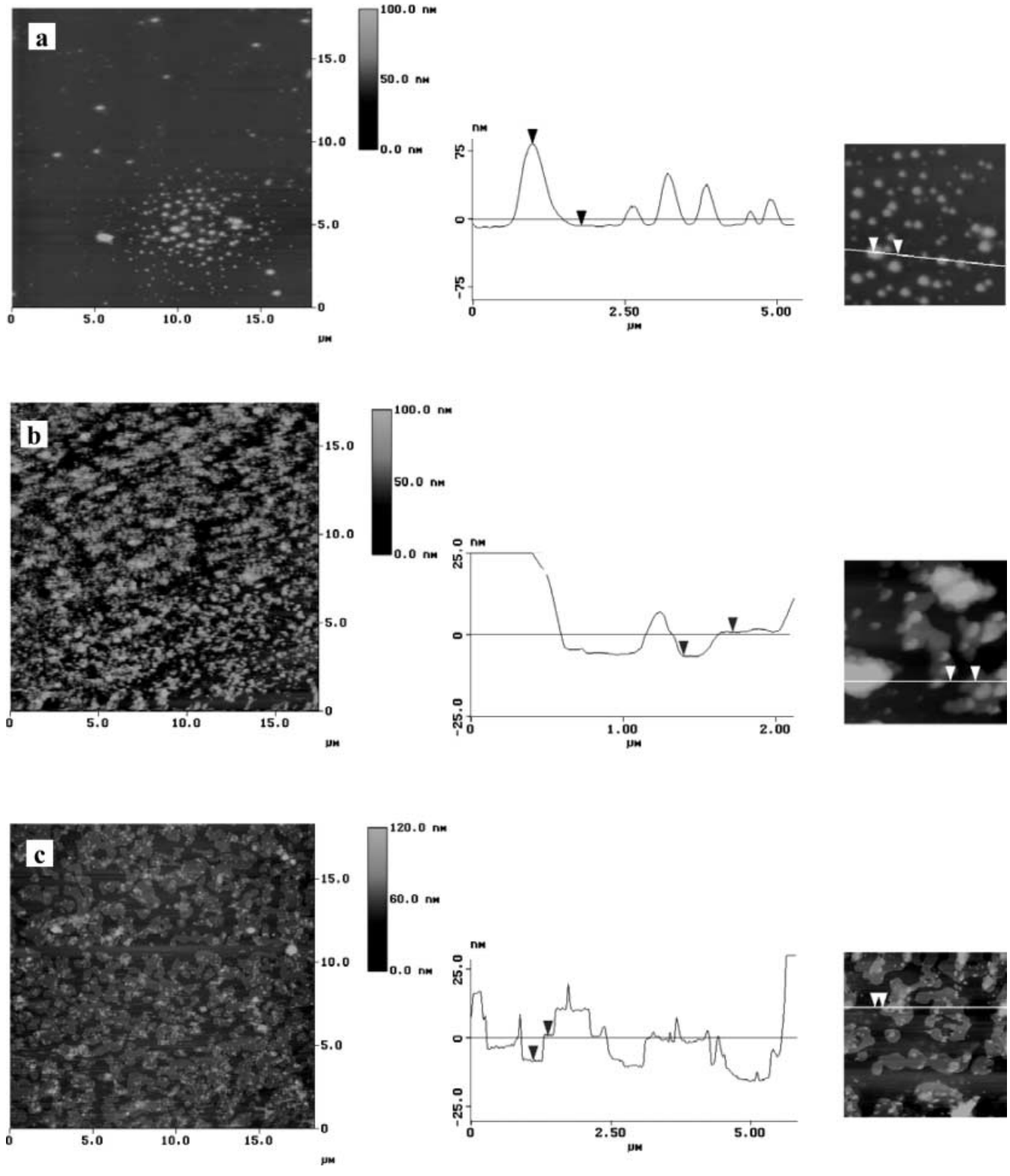
Fig. 5a–c SFM images (contact mode) of LB films during the insertion of DPPC/DPPG (4:1) vesicles. Films at an initial surface pressure of 30 mN/m were transferred onto mica sheets covered with a DPPC monolayer at a temperature of 20 °C. The *black lines* in the smaller images on the *right* show the region of the line trace. The *arrows* on the height trace show the approximate height difference. **a** DPPC/DPPG (4:1); **b** DPPC/DPPG/SP-C (lipid ratio 4:1, protein content 0.4 mol%) (the height difference between the arrows is 7–8 nm); **c** DPPC/DPPG/SP-B (lipid ratio 4:1, protein content 0.2 mol% per dimer) (the height difference between the marked region of the line scan is about 10 nm)

Scanning force microscopy

In order to study the structures formed at the air/water interface during the insertion process in situ, SFM under water was performed. With this aim in view, a precoated hydrophobic DPPC monolayer on a mica plate as substrate was dipped through the lipid or lipid/protein monolayer at the air/water interface. The resulting bilayer with the lipid headgroups exposed to the subphase served as the docking site for the vesicles. The images obtained from these LB transfers in an aqueous environment are presented in Fig. 5, together with line traces along the black lines in the smaller images on the right.

As can be seen from experiments performed in the absence of either SP-B or SP-C, few vesicles merely adhere to the surface but do not rupture (Fig. 5a). Vesicles at the surface remain intact and are slightly flattened owing to the adhesion mediated by calcium ions and the forces exerted by the cantilever. They are only loosely bound and can be laterally moved with the cantilever. The corresponding height profile reveals that the structures are of variable height (20–90 nm). Since vesicles with a diameter of 50 nm were prepared, calcium-mediated aggregation and subsequent fusion must have occurred, leading to larger structures. Removal of calcium by EDTA as chelator results in a drastic reduction of vesicle material adsorbed on the surface (data not shown).

However, the presence of SP-B or SP-C tremendously increased the amount of adsorbed material, leading to a complete coverage with vesicles, which were partly aggregated and fused (Fig. 5b, c). To a great extent, the material was only loosely bound to the membrane and could easily be moved around with the tip of the cantilever. Interestingly, below this loosely bound material, regions of tightly packed stacks were observable in the case of SP-C as well as in the presence of SP-B. Similar structures have been found in the plateau region of the pressure-area isotherms, representing



collapse of a SP-C-containing monolayer (Amrein et al. 1997; von Nahmen et al. 1997b; Galla et al. 1998) and in monolayers containing negatively charged lipids and SP-B (Takamoto et al. 2001). It seems that these stacks are presumably formed due to strong adhesive forces, leading to strong flattening of the vesicles which eventually results in rupture. The structures are best described as bilayer stacks exhibiting an incremental height of about 7–9 nm for the SP-C-containing monolayer (Fig. 5b) and 8–10 nm in the case of SP-B-containing monolayers (Fig. 5c). This marginal height difference is probably due to the different

thickness of water layers between the ruptured vesicle and the monolayer. Remarkably, the number of stacked bilayers is greater in the case of SP-B-containing monolayers compared to those supplemented by SP-C.

Discussion

Pulmonary surfactant proteins SP-B and SP-C are known to be a prerequisite for fast replenishment of phospholipids into preformed monolayers at the air/water interface (Oosterlaken-Dijksterhuis et al. 1991a, 1991b; Creuwels et al. 1995; Nag et al. 1996, 1998; Cruz et al. 1997; Walters et al. 2000). The process, which can basically be treated as a two-step mechanism, consists of initial adsorption of vesicles on the monolayer and subsequent rupture and integration facilitated by SP-B and SP-C.

In order to explain the differences in protein-mediated insertion kinetics presented in this study, one must not only consider the dimensions of the proteins but also their ability to interact with negatively charged lipids and to induce phase separation in the monolayer. This behavior was studied earlier by film balance measurements and fluorescence microscopy at the air/water interface (Krol et al. 2000a, 2000b). Although we used pure water in this previous study, the results correspond well to those obtained from a buffered subphase containing calcium ions (data not shown). For a better understanding of the following discussion of the protein-mediated insertion kinetics, some of the film balance and fluorescence microscopy results will be discussed briefly.

Pressure/area isotherms of SP-B-containing monolayers on a buffered subphase with calcium exhibit the same two small plateau regions at a surface pressure of 25–28 mN/m and 40 mN/m as on a pure water subphase (Krol et al. 2000a). It is generally concluded from these isotherms that SP-B fluidizes the monolayer and therefore leads to an enhanced compressibility over the whole area range (Lipp et al. 1996; Lee et al. 1998; Krol et al 2000a). The effect of SP-C on the lipid film is less pronounced at low surface pressures: the isotherms of monolayers containing SP-C display the typical low compressibility of a lipid mixture without protein. However, at surface pressures above 50 mN/m the influence of SP-C on the monolayer characteristics is tremendous: an extended plateau region occurs at 50–52 mN/m owing to a reversible removal of lipids and peptide from the monolayer (Post et al. 1995; von Nahmen et al. 1997a; Bourdos et al. 2000; Krol et al. 2000a). These finding were corroborated by fluorescence microscopy (Krol et al. 2000a, 2000b). The surfactant proteins seem to act as defects in the monolayer and thus prevent the continuous growth of liquid-condensed domains as they occur in neat lipid monolayers. As a result, smaller liquid-condensed domains were visible when SP-B and SP-C were present in the monolayer. It is very likely that SP-B, being the larger protein, covers a more extended molecular area than does SP-C and therefore fluidizes the monolayer (Lee et al. 1997, 1998; Lipp et al. 1996, 1997; Nag et al. 1998). This behavior of surfactant proteins in monolayers is important to interpret the differences in insertion kinetics.

The kinetic studies performed at 37 °C revealed that lipid films containing either SP-B or SP-C had to be compressed to initial surface pressures well above 30 mN/m in order to allow insertion of lipid material. This observation does not correspond to previous results (Oosterlaken-Dijksterhuis et al. 1991a, 1991b), where incorporation of phospholipids was already detected at a threshold value of 20 mN/m. The reason for this discrepancy in insertion kinetics is probably the difference in monolayer composition. Oosterlaken-Dijksterhuis et al. performed their experiments with 30 mol% egg-PG, 0.4 mol% SP-B and 3.5 mol% SP-C in the monolayer, whereas we chose an amount of 20 mol% saturated PG, 0.2 mol% SP-B and 0.4 mol% SP-C, corresponding to the experimental conditions of previous studies (Post et al. 1995; Amrein et al. 1997; von Nahmen et al. 1997a; Galla et al. 1998; Krol et al. 2000a, 2000b) and the relative amount of various PGs found in bovine surfactant to DPPC (Possmayer et al. 1984). At 37 °C we observed almost identical insertion rates at a surface pressure of 30 mN/m for both surfactant proteins, whereas at higher initial surface pressures considerably faster SP-B-induced vesicle insertion was detected. This corresponds to previous studies reporting a four-fold higher activity by weight for SP-B than SP-C (Oosterlaken-Dijksterhuis et al. 1991b).

In order to gain more information on the protein induced insertion process, the temperature was reduced to 20 °C. At this temperature the spread monolayers had to be compressed to initial surface pressures of at least 15 mN/m to enable vesicle insertion. In contrast to the measurements at 37 °C, considerable differences in insertion kinetics between monolayers containing either SP-B or SP-C were observed. The SP-B-induced vesicle insertion was characterized by a fast sigmoidal increase in surface pressure after injection of vesicles into the subphase and a typical decrease in the slope of pressure/time diagrams beyond 40 mN/m. Interestingly, we found a maximum insertion rate for monolayers containing SP-B at an initial surface pressure of 25 mN/m. This may be a trade-off value in which highest fluidity and surface charge density are achieved. In contrast to SP-B, SP-C-containing monolayers showed a sigmoidal course characterized by a long lag-phase (300–1500 s) up to 25 mN/m. This delay continuously decreased with increasing surface pressure. In contrast to SP-B, SP-C-induced vesicle insertion did not show a maximum insertion rate but increased continuously with increasing surface pressure. Comparing the quantified insertion rates of SP-B- and SP-C-induced vesicle insertion leads to the conclusion that SP-B is more effective in promoting the insertion of lipids from vesicles in the subphase, especially at lower initial surface pressures. The insertion rates for SP-B-containing monolayers are at least

three-fold higher. A possible reason for this higher activity of SP-B might be that it has a stronger influence on the morphology of the monolayer, rendering it more fluid (see above). It might lead to a more pronounced lateral phase separation than does SP-C, because it interacts stronger with negatively charged lipids and induces the formation of SP-B/DPPG-rich regions with enhanced surface charge density. The only exception was found at 40 mN/m, where the activity of SP-C was twice as high as the activity of SP-B. This may be explained by the exclusion of SP-B at higher surface pressures, an observation that has already been reported for monolayers at the air/water interface that have been compressed to surface pressures between 40 and 45 mN/m (Taneva and Keough 1994; Pastrana-Rios et al. 1995; Krol et al. 2000a). Regarding the insertion kinetics, one can assume that this exclusion process begins at 25 mN/m and ends at 45 mN/m. This finding is in good agreement with data from the literature (Pastrana-Rios et al. 1994, 1995; Taneva and Keough 1994; Krol et al. 2000a).

SP-C, however, is known to remain in the monolayer up to surface pressures of about 55 mN/m before it is squeezed out from the interface, forming stacked bilayer structures underneath the monolayer that are composed of lipids and proteins (von Nahmen et al. 1997a, 1997b; Galla et al. 1998; Krol et al. 2000b; Bourdos et al. 2000; Takamoto et al. 2001). This might be the reason why a continuous increase in insertion kinetics with rising initial surface pressure is observable. Only at 50 mN/m is vesicle insertion abolished. It is assumed that the α-helical part of SP-C spans the hydrophobic part of the lipid bilayers and the remaining residues interact with the lipid head-groups (Johansson et al. 1994). These results have led to the conclusion that the physiological role of SP-C is to prevent an irreversible film collapse by building a surfactant reservoir adjacent to the monolayer and to guarantee a closed surface coverage during inhalation (Galla et al. 1998). Since SP-C is stable in the monolayer up to high surface pressures it is able to continuously enhance the insertion of phospholipids from an aqueous subphase also at high initial surface pressure.

By employing SFM it was possible to visualize the binding of phospholipid vesicles to the monolayer that was transferred to a hydrophobic substrate (DPPC on mica). As the experiments with a pure lipid monolayer showed, binding of vesicles was mediated by calcium ions. When the divalent cations were removed from the subphase, less material was found to be attached to the monolayer.

In the presence of surfactant proteins, however, we not only observed a higher amount of adsorbed material that was loosely bound to the monolayer, we also found stacked bilayers with a height of 7–10 nm over an extended area that were tightly bound to the surface. Removal of divalent cations from the subphase resulted in less material attached to the monolayer. This is consistent with the observations by Oosterlaken-Dijksterhuis et al. (1991b), who found that vesicles do not desorb easily from protein-containing monolayers, even after Ca^{2+} depletion of the subphase, and that eight times as much phospholipid was bound to the monolayers as was inserted into the monolayer.

SFM studies reveal that SP-B and SP-C induce vesicle insertion in a similar fashion: the adsorption of vesicular material is stabilized and the strong adhesion leads to flattening and eventually rupture of the vesicles, resulting in efficient replenishment of lipids into the monolayer.

Besides fluidity, negative charges in conjugation with Ca^{2+} ions are found to be of major importance for fast respreading of lipid material to the air/liquid interface. The importance of anionic lipids for the insertion process, especially in combination with SP-B, which interacts more avidly with negatively charged phospholipids as SP-C, has been recognized by others (Baatz et al. 1990; Pérez-Gil et al. 1995; Cruz et al. 1998). A previous study revealed that anionic lipids are especially important for the initial process of vesicle adsorption, not only to a clean surface but also to a preformed monolayer at the air/water interface (Walters et al. 2000). In their study, however, surfactant proteins were present in the vesicles and anionic lipids in the monolayer, while in our study surfactant proteins were solely in the monolayer. Furthermore, we seek to confirm the assumption that it does not matter whether anionic lipids are in vesicles or in a monolayer.

As our results indicate, the insertion of vesicular material was indeed faster with increasing DPPG concentration in the vesicles, although negatively charged lipids in vesicles were not absolutely necessary for the insertion process. These results confirm the assumption made by Walters et al. (2000) that calcium ions modulate hydration forces and entropic effects not only of negatively charged but also of neutral lipids and thus reduce a thermodynamic barrier that opposes the close approach of two bilayers. This influence should be more pronounced when negatively charged lipids are present in both bilayers and calcium in the subphase. Therefore increasing the amount of DPPG in vesicles should lead to steeper initial slopes of protein-mediated insertion kinetics, an assumption that was confirmed in our study.

The fact that the influence of DPPG was more pronounced in the presence of SP-B can only be attributed to a preferential interaction of SP-B with DPPG, leading to a SP-B/DPPG-enriched phase in the monolayer which considerably enhances insertion of phospholipids. As Pérez-Gil et al. (1995) showed, SP-B expresses a higher affinity for PG^{-} than for $PC^{\pm}$ at low and at high ionic strength, whereas SP-C did not show any preferential interaction for PG. The efficacy of SP-C therefore most probably lies in its hydrophobic properties rather than in specific interactions with the lipid polar groups.

Conclusions

From our results we conclude that replenishment of lipids to the air/liquid interface can roughly be described

by a two-step mechanism. In the first step, vesicles adsorb on the monolayer modulated by surfaces charges, surfactant proteins and Ca^{2+} ions in solution. We found that negatively charged lipids in the monolayer as well as in the vesicles are most relevant for rapid respreading. It is conceivable that Ca^{2+} ions form bridges to connect PG molecules in the monolayer with those in liposomes and therefore enhance vesicle adsorption. However, when negatively charged lipids are only present in one of the two opposing membranes, Ca^{2+} ions seem to modulate the hydration state of neutral phospholipids, thus reducing the thermodynamic barrier for the close approach of two bilayers.

The second step involves rupture and integration, which apparently is facilitated by fluidization of the monolayer accomplished by surfactant proteins. It was shown that SP-B fluidizes the monolayer at low to intermediate surface pressure, while SP-C efficiently increases compressibility at higher surface pressure. SFM images reveal that stacked bilayers adjacent to the monolayer are formed solely in the presence of surfactant proteins, supporting the hypothesis that surfactant proteins enhance adhesion of vesicles and facilitate integration.

References

Amrein M, Nahmen A von, Sieber M (1997) A scanning force- and fluorescence light microscopy study of the structure and function of a model pulmonary surfactant. Eur Biophy J 26:349–357

Baatz JE, Elledge B, Whitsett JA (1990) Surfactant protein SP-B induces ordering at the surface of model membrane bilayers. Biochemistry 29:6714–6720

Bourdos N, Kollmer F, Benninghoven A, Ross M, Sieber M, Galla H-J (2000) Analysis of lung surfactant model systems with time-of-flight secondary ion mass spectrometry. Biophys J 79: 357–369

Creuwels LAJM, Boer EH, Demel RA, Golde LMG van, Haagsman HP (1995) Neutralization of the positive charges of surfactant protein C. J Biol Chem 270:16225–16229

Crouch EC (1998) Structure, biologic properties, and expression of surfactant protein D (SP-D). Biochim Biophys Acta 1408:278–289

Cruz A, Casals C, Keough KMW, Perez-Gil J (1997) Different modes of interaction of pulmonary surfactant protein SP-B in phosphatidylcholine bilayers. Biochem J 327:133–138

Cruz A, Marsh D, Perez-Gil J (1998) Rotational dynamics of spin-labeled surfactant-associated proteins SP-B and SP-C in dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylglycerol bilayers. Biochim Biophys Acta 1415:125–134

Curstedt T, Jörnvall H, Robertson B, Bergman T, Berggren P (1987) Two hydrophobic low-molecular-mass protein fractions of pulmonary surfactant: characterization and biophysical activity. Eur J Biochem 168:255–262

Galla H-J, Bourdos N, Nahmen A von, Amrein M, Sieber M (1998) The role of pulmonary surfactant protein C during the breathing cycle. Thin Solid Films 327–329:632–635

Goerke J, Clements JA (1986) In: Macklem, Mead (eds) Handbook of physiology. American Physiological Society, Washington, pp 247–261

Hall SB, Venkitaraman AR, Whitsett JA, Holm BA, Notter RH (1992) Importance of hydrophobic apoproteins as constituents of clinical exogenous surfactants. Am Rev Respir Dis 145:24–30

Hawgood S, Derrick M, Poulain F (1998) Structure and properties of surfactant protein B. Biochim Biophys Acta 1408:150–160

Johansson J (1998) Structure and properties of surfactant protein C. Biochim Biophys Acta 1408:161–172

Johansson J, Szyperski T, Curstedt T, Wüthrich K (1994) The NMR structure of the pulmonary surfactant-associated polypeptide SP-C in an apolar solvent contains a valyl-rich α-helix. Biochemistry 33:6015–6023

Krol S, Ross M, Sieber M, Künneke S, Galla H-J, Janshoff A (2000a) Formation of three dimensional protein-lipid-aggregates in monolayer films induced by surfactant protein B. Biophys J 79:904–918

Krol S, Janshoff A, Ross M, Galla H-J (2000b) Structure and function of surfactant protein B and C in lipid monolayers: a scanning force microscopy study. Phys Chem Chem Phys 2:4586–4593

Lee KYC, Lipp MM, Zasadzinski JA, Waring AJ (1997) Effects of lung surfactant specific protein SP-B and model SP-B peptide on lipid monolayers at the air/water interface. Colloids Surf A 128:225–242

Lee KYC, Lipp MM, Zasadzinski JA, Waring AJ (1998) Direct observation of phase and morphology changes induced by lung surfactant protein SP-B in lipid monolayers via fluorescence, polarized fluorescence, Brewster angle and atomic force microscopies. SPIE 3273:115–133

Lipp MM, Lee KYC, Zasadzinski JA, Waring AJ (1996) Phase and morphology changes in lipid monolayers induced by SP-B protein and its amino-terminal peptide. Science 273:1196–1199

Lipp MM, Lee KYC, Waring AJ, Zasadzinski JA (1997) Fluorescence, polarized fluorescence, and Brewster angle microscopy of palmitic acid and lung surfactant protein B monolayers. Biophys J 72:1–21

McCormack FX (1998) Structure, processing and properties of surfactant protein A. Biochim Biophys Acta 1408:109–131

Nag K, Pérez-Gil J, Cruz A, Rich NH, Keough KMW (1996) Spontaneous formation of interfacial lipid-protein monolayers during adsorption from vesicles. Biophys J 71:1356–1363

Nag K, Pérez-Gil J, Ruano ML, Worthman LA, Stewart J, Casals C, Keough KMW (1998) Phase transitions in films of lung surfactant at the air/water interface. Biophys J 74:2983–2995

Nahmen A von, Post A, Galla H-J, Sieber M (1997a) The phase behavior of lipid monolayers containing pulmonary surfactant protein C studied by fluorescence light microscopy. Eur Biophys J 26:359–369

Nahmen A von, Schenk M, Sieber M, Amrein M (1997b) The structure of a model pulmonary surfactant as revealed by scanning force microscopy. Biophys J 72:463–469

Oosterlaken-Dijksterhuis MA, Haagsman HP, Golde LMG van, Demel RA (1991a) Interaction of lipid vesicles with monomolecular layers containing lung surfactant proteins SP-B or SP-C. Biochemistry 30:8276–8281

Oosterlaken-Dijksterhuis MA, Haagsman HP, Golde LMG van, Demel RA (1991b) Characterization of lipid insertion into monomolecular layers mediated by lung surfactant proteins SP-B and SP-C. Biochemistry 30:10965–10971

Oosterlaken-Dijksterhuis MA, Eijk M van, Golde LMG van, Haagsman HP (1992) Lipid mixing is mediated by the hydrophobic surfactant protein SP-B but not by SP-C. Biochim Biophys Acta 1110:45–50

Pastrana-Rios B, Flach CR, Brauner JW, Mautone AJ, Mendelsohn R (1994) A direct test of the ''squeeze-out'' hypothesis of lung surfactant function. External reflection FT-IR at the air/water interface. Biochemistry 33:5121–5127

Pastrana-Rios B, Taneva S, Keough KMW, Mautone AJ, Mendelsohn R (1995) External reflection absorption infrared spectroscopy study of lung surfactant protein SP-B and SP-C in phospholipid monolayers at the air/water interface. Biophys J 69:2531–2540

Pérez-Gil J, Keough KMW (1998) Interfacial properties of surfactant proteins. Biochim Biophys Acta 1408:203–217

Pérez-Gil J, Casals C, Marsh D (1995) Interactions of hydrophobic lung surfactant proteins SP-B and SP-C with dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylglycer-

ol bilayers studied by electron spin resonance spectroscopy. Biochemistry 34:3964–3971

Possmayer F, Yu S-H, Weber JM, Harding PGR (1984) Pulmonary surfactant. Can J Biochem Cell Biol 62:1121–1133

Post A, Nahmen A von, Schmitt M, Ruths J, Riegler H, Sieber M, Galla H-J (1995) Pulmonary surfactant protein C containing lipid films at the air/water interface as a model for the surface of lung alveoli. Mol Membr Biol 12:93–99

Reid KBM (1998) Interactions of surfactant protein D with pathogens, allergens and phagocytes. Biochim Biophys Acta 1408:290–295

Takamoto DY, Lipp MM, Nahmen A von, Lee KYC, Waring AJ, Zasadzinski JA (2001) Interaction of lung surfactant proteins with anionic phospholipids. Biophys J 81:153–169

Taneva S, Keough KMW (1994) Pulmonary surfactant proteins SP-B and SP-C in spread monolayers at the air/water interface: I. Monolayers of pulmonary surfactant protein SP-B and phospholipids. Biophys J 66:1137–1148

Walters RW, Jenq RR, Hall SB (2000) Distinct steps in the adsorption of pulmonary surfactant to an air-liquid interface. Biophys J 78:257–266