The Role of Surfactant Proteins in DPPC Enrichment of Surface Films

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ABSTRACT A pressure-driven captive bubble surfactometer was used to determine the role of surfactant proteins in refinement of the surface film. The advantage of this apparatus is that surface films can be spread at the interface of an air bubble with a different lipid/protein composition than the subphase vesicles. Using different combinations of subphase vesicles and spread surface films a clear correlation between dipalmitoylphosphatidylcholine (DPPC) content and minimum surface tension was observed. Spread phospholipid films containing 50% DPPC over a subphase containing 50% DPPC vesicles did not form stable surface films with a low minimum surface tension. Addition of surfactant protein B (SP-B) to the surface film led to a progressive decrease in minimum surface tension toward 1 mN/m upon cycling, indicating an enrichment in DPPC. Surfactant protein C (SP-C) had no such detectable refining effect on the film. Surfactant protein A (SP-A) had a positive effect on refinement when it was present in the subphase. However, this effect was only observed when SP-A was combined with SP-B and incubated with subphase vesicles before addition to the air bubble containing sample chamber. Comparison of spread films with adsorbed films indicated that refinement induced by SP-B occurs by selective removal of non-DPPC lipids upon cycling. SP-A, combined with SP-B, induces a selective adsorption of DPPC from subphase vesicles into the surface film. This is achieved by formation of large lipid structures which might resemble tubular myelin.

INTRODUCTION

Pulmonary surfactant is a mixture of lipids and specific proteins that is secreted by the epithelial type II cells into the alveolar space. Its main function is to reduce the surface tension (γ) at the air/liquid interface in the lung. This is needed to lower the work of breathing and to prevent alveolar collapse at end-expiration.

Surfactant proteins have been shown to be critical for effective physiological function within the lung. In vitro studies show that adsorption of lipids into an air/liquid interface is greatly enhanced when small amounts of the hydrophobic surfactant proteins B or C (SP-B and SP-C) are added (Oosterlaken-Dijksterhuis et al., 1991; Pérez-Gil et al., 1992; Schürch et al., 1994; Krill and Gupta, 1994). The hydrophilic surfactant protein A (SP-A) does not have this effect by itself but seems to enhance the effect of SP-B (Cockshutt et al., 1990). The importance of SP-B and SP-C is also apparent from treatment of the neonatal and acute respiratory distress syndrome. Administration of exogenous surfactants containing SP-B or SP-C or synthetic variants thereof shows superior results compared to that of protein-free surfactant preparations (Revak et al., 1996; Cochrane et al., 1996; Hawgood et al., 1996; da Costa et al., 1999; Hall et al., 1992). Besides that, lethal respiratory distress is caused by SP-B deficiency in humans (Nogee et al., 1994; Tredano et al., 1999) and in SP-B knockout mice (Tokieda et al., 1997). Surprisingly, SP-C knockout mice, which have recently been generated, seem to have normal oxygenation (Glasser, 1999).

Dipalmitoylphosphatidylcholine (DPPC) contributes approximately 40 to 50% to the total phospholipid pool of pulmonary surfactant (Kahn et al., 1995; Brouwers et al., 1998; Bernhard et al., 1997). Unsaturated phosphatidylcholine (PC), phosphatidylglycerol (PG), neutral lipids, and small traces of other lipids complete the composition. Within the lung, the surface film, which consists of these lipid components, has to withstand high surface pressures at low lung volumes (Schürch, 1982). It is generally believed that this is achieved by enrichment of the surface film in DPPC. Upon compression, DPPC can pack into a gel phase that can resist these high surface pressures (Watkins, 1968). The exact mechanism by which this enrichment in DPPC occurs is unknown, but there are several indications that surfactant proteins have an active role in this process.

Two main ways by which DPPC enrichment can occur are selective adsorption of DPPC and selective removal of non-DPPC lipids. Indications of the existence of both mechanisms have been described. The evidence for selective DPPC adsorption stems from studies in the captive bubble surfactometer (CBS; Schürch et al., 1992). In these studies, a surface film was formed at the interface of an air bubble by adsorption of material from the surfactant-containing subphase. After adsorption was completed, the air bubble required less area reduction to reach very low surface tensions than could be expected from the lipid composition of the surfactant in the subphase. This indicated that DPPC was specifically adsorbed from the subphase to the air/liquid interface of the bubble. Indications for the selective removal of non-DPPC lipids stem mostly from localization studies in spread lipid/protein films on a Wilhelmy surface balance (von Nahmen et al., 1997b; Nag et al., 1997). Upon

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compression of such films, gel-like phases surrounded by fluid phases developed and surfactant proteins and unsaturated lipids localized in these fluid phases. When more pressure was applied to these films, collapse occurred and the fluid phases were squeezed out of the interface (Pastrana et al., 1994; von Nahmen et al., 1997a).

The role of other lipid components is unclear. Several in vitro studies have indicated that the complete set of surfactant lipids has superior activity over simplified lipid mixtures (Wang et al., 1995; Ingenito et al., 1999). Removal of, for example, the neutral lipids resulted in lowered surface activity. The use of simple binary or ternary lipid mixtures supplemented with one or more surfactant proteins can lead to assigning specific functions to specific lipids instead of whole groups of lipids (like the above-mentioned neutral lipids). A nice example is a recent study, which showed that the interaction of SP-B with the anionic phospholipid PG is required for efficient refining of the surfactant film (Nag et al., 1999). When PG was replaced by unsaturated PC, no refining was observed. Other studies, with sometimes contradictory results, on the role of cholesterol and palmitic acid have been conducted (Cockshutt et al., 1991; Lema and Enhorning, 1997; Palmblad et al., 1999; Yu and Possmayer, 1994; Palmer et al., 1997; Taneva and Keough, 1997).

The pressure-driven CBS that we use in our study has several advantages over other model systems used to test the activity of natural or model surfactants (Putz et al., 1994). The CBS offers a leakproof system in which the surfactant samples can be tested dynamically. In addition, films of known lipid/protein composition can be spread at the interface of the air bubble (Putz et al., 1998), contrary to most systems where the films at the interface are adsorbed from surfactant material in the subphase. Because the adsorption mechanisms can differ for different surfactant samples, the starting situations, using adsorbed films, are not comparable to each other when the bubble is cycled (expanded and compressed to resemble breathing). Finally, the composition of the lipids and proteins in the subphase can be made different from the composition of the surface film.

In order to determine the role of the surfactant proteins we used simple ternary and binary lipid mixtures of DPPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) (POPG). In most studies where simple lipid mixtures are used, relatively high amounts of 70–80% DPPC are incorporated in the mixture. If an enrichment of the monolayer in DPPC occurs, only a relatively small amount of non-DPPC lipids has to be removed. In earlier studies using the CBS, we observed that refinement did indeed occur and was completed after one or two cycles (Putz et al., 1999; Veldhuizen et al., 1999). In this study, DPPC was gradually substituted by POPC until a limiting effect of this substitution in the surface activity of the sample was observed. Subsequently, we determined the effect of the surfactant proteins on refinement of the monolayer, i.e., enriching it in DPPC. The studies suggest that SP-B, but not SP-C, has a role in refinement of the monolayer in DPPC by the selective squeeze-out mechanism. SP-A enhances the selective adsorption of DPPC, but only when SP-B is present. No cooperation between SP-A and SP-C was found.

EXPERIMENTAL PROCEDURES

Materials

DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine), POPC, and POPG were obtained from Avanti Polar Lipids (Alabaster, AL). HEPES was from Life Technologies (Paisley, UK). EDTA, calcium chloride ($CaCl_2$), chloroform ($CHCl_3$), and methanol (MeOH) were from Baker Chemicals B.V. (Deventer, The Netherlands). Organic solvents were distilled before use. Porcine SP-B and SP-C were isolated from lung lavage according to standard procedures (Oosterlaken-Dijksterhuis et al., 1991). Human SP-A was isolated from human lavage as described previously (Haagsman et al., 1987).

Vesicle preparation

Small unilamellar vesicles were prepared as follows. Lipids from stock solutions in $CHCl_3$/MeOH were dried under a continuous stream of nitrogen at room temperature. When SP-B or SP-C were required in the vesicles, these were added and mixed with the lipids from a stock solution in $CHCl_3$/MeOH. The resulting dry lipid/peptide film was rehydrated by adding CBS buffer (see below), vortexing, and incubating at 55°C for 15 min. The multilamellar vesicles thus formed were sonicated for 2×1 min at 55°C with a 10-s interval. The vesicles were cooled to 37°C and used immediately. When SP-A was required in the vesicles, it was added from a stock solution in 5 mM Tris after sonication of the vesicles. In all experiments the same surfactant protein concentrations were used: 3 mol% SP-C and 0.75 mol% SP-B incorporated in vesicles and 0.1 μg SP-A added to the vesicles.

Biochemical analysis

To check the purity of the surfactant proteins, protein electrophoresis was performed by one-dimensional Tricine/sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Schägger and von Jagow, 1987). After electrophoresis, proteins were stained with silver stain (Bio-Rad Laboratories, Richmond, CA). The purity of phospholipids was checked by thin layer chromatography using $CHCl_3$:MeOH:H_2O (65:35:4, v:v:v) as mobile phase. Concentration of phospholipid stock solutions was determined using a phosphorus assay (Rouser et al., 1970).

Captive bubble surfactometry

The capability of the surfactant proteins to insert lipids into the air/liquid interface and to enrich the interface layer in DPPC was determined using a pressure-driven CBS (Putz et al., 1994). A bubble (0.5 cm^2) was formed in subphase buffer (140 mM NaCl, 10 mM HEPES, 0.5 mM EDTA, 2.5 mM $CaCl_2$, pH 6.9) by injecting air (28.5 μl) into the sample chamber at 1.0 absolute atmospheric pressure (ata) and 37°C. Two methods of surface film formation were used: film spreading and adsorption from the subphase.

Film spreading

Stock solutions of DPPC:POPC:POPG with or without proteins were prepared in $CHCl_3$:MeOH (1:1, v:v). From this stock solution, 0.05 μl

(0.25 nmol lipids) was spread at the air/water interface using a glass syringe. This resulted in a surface tension of approximately 22 mN/m (equilibrium surface tension). In all experiments, 3 mol% SP-C or 0.75% SP-B was used, which corresponds to the concentration resulting in maximum surface activity in spread films (unpublished results). The subphase was stirred for 30 min to enhance desorption of solvent from the surface. Subsequently, 100 μl of small unilamellar vesicles were injected into the subphase (final concentration 1 μmol PL/ml) and stirring was continued for another 15 min. This subphase phospholipid concentration results in maximum surface activity of spread films (unpublished results). The bubble area was increased by sudden lowering of the pressure inside the sample chamber to 0.5 ata for 10 s. This resulted in approximately a twofold area increase (depending slightly on the final surface tension). Subsequently, the bubble was cycled five times in 1 min between two preset pressure values of 0.5 ata and 2.8 ata, resulting in a dynamic compression and expansion of the air bubble. After the cycling procedure, another 15 min adsorption time was allowed, followed by a second series of five cycles. A video camera continuously monitored the shape of the bubble, from which the surface tension values were calculated (Putz et al., 1998).

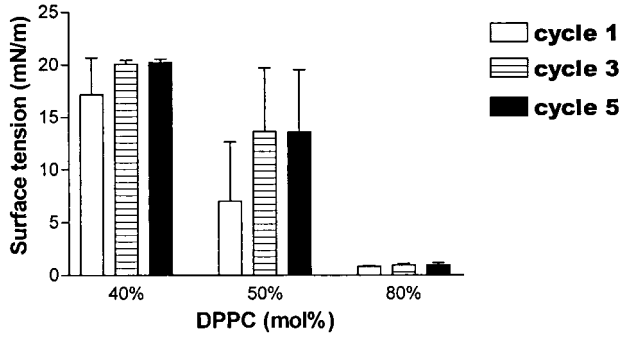

FIGURE 1 Effect of DPPC content on the minimum surface tension (γ_{min}) of spread pure lipid films. The γ_{min} of the first (*white bars*), third (*hatched bars*), and fifth compression (*black bars*) are shown. The composition of the subphase vesicles was the same as that of the spread film. Values shown are the averages of at least three separate experiments ± SEM.

Film adsorption

Small unilamellar vesicles of DPPC: POPC:POPG with or without proteins were prepared as described and injected directly into the sample chamber. The final concentration in the subphase was 1 μmol PL/ml. The material was allowed to adsorb to the interface of the bubble for 15 min, followed by the same cycling and re-adsorption period described above.

All lipid samples used, vesicles and spread films, contained 20% POPG, whereas the amount of DPPC and POPC was varied. The composition will be referred to only by the DPPC content. For example: 50% DPPC vesicles have a composition of DPPC:POPC:POPG (50:30:20, mol:mol:mol).

RESULTS

A clear effect of the percentage of DPPC on the γ_{min} values of protein-free spread films, on a subphase containing liposomes with the same composition, was observed. In previous studies (Veldhuizen et al., 1999, 2000; Putz et al., 1998, 1999) 80% DPPC was used as the standard lipid mixture, which results in near-zero minimum surface tensions during cycling. However, when lowered amounts of DPPC were present, the films caused higher minimum surface tensions, as shown in Fig. 1. At 50% DPPC, a γ_{min} of either 2 mN/m or 20 mN/m was observed for separate experiments. No γ_{min} values in between 2 and 20 mN/m were detected. This all-or-nothing behavior is specific for the protein-free surface films and, apparently, the 50% DPPC mixture is at the limit of the ability to create very low γ_{min}. Small differences during the experiment determine whether a very low or high γ_{min} is achieved upon compression. The increase in average minimum surface tension upon cycling of the film should therefore be interpreted as an increase in the number of films that are not able to create stable films with near-zero surface tensions. When 40% DPPC is present, near-zero values are not reached after the first cycle in any separate experiment.

When the concentrations of DPPC were varied, no significant differences were observed for the maximum surface tensions (γ_{max}) of SP-B or SP-C containing surface films (Table 1). This showed that the ability of SP-B and SP-C to insert lipids into the surface film upon expansion of the air bubble is not affected by the percentage of DPPC.

A clear refinement of the surface film was observed when SP-B was present in films containing 50% DPPC. Upon the

TABLE 1 Maximum and minimum surface tensions (in mN/m) of spread films during cycling

Lipid Composition	No protein		3% SP-C		0.75% SP-B	
	γ_{max}	γ_{min}	γ_{max}	γ_{min}	γ_{max}	γ_{min}
40% DPPC						
1st cycle	43.6 ± 1.6	17.2 ± 3.5	not done	not done	21.6 ± 0.4	13.9 ± 4.2
5th cycle	54.5 ± 4.4	20.2 ± 0.4			30.2 ± 3.9	13.0 ± 5.8
50% DPPC						
1st cycle	40.9 ± 5.6	7.0 ± 5.6	21.4 ± 0.9	10.2 ± 3.8	22.1 ± 0.2	6.9 ± 5.2
5th cycle	58.7 ± 1.7	13.6 ± 5.9	39.5 ± 5.8	19.7 ± 0.6	32.8 ± 1.6	2.3 ± 1.6
80% DPPC						
1st cycle	46.5 ± 1.1	0.8 ± 0.1	21.9 ± 0.4	0.5 ± 0.1	23.5 ± 0.7	0.6 ± 0.1
5th cycle	50.5 ± 2.3	1.0 ± 0.2	31.3 ± 2.3	0.6 ± 0.1	31.4 ± 0.3	0.5 ± 0.1

Values represent the mean ± SEM.
The composition of the subphase vesicles was the same as that of the spread film.

first compression a minimum surface tension of approximately 7 mN/m was achieved which gradually decreased toward stable films with γ_{min} close to 1 mN/m (Fig. 2). This reflects an enrichment in DPPC of the surface film at the interface. At an even lower DPPC concentration of 40% no refinement occurred anymore and γ_{min} values of approximately 13 mN/m at every compression were observed (not shown, but comparable to the experiment described below where, in addition to its presence in the surface film, SP-B was also present in the subphase vesicles [Fig. 5, open circle]).

No refining mechanism was observed when SP-C was present in a 50% DPPC film (Fig. 2). During cycling the γ_{min} rose from approximately 10 mN/m, which is comparable to the value of SP-B in this lipid mixture at the first compression, to 19 mN/m. This indicates a loss of material during cycling, probably due to overcompression of the film. When different combinations of film lipid composition and subphase vesicles lipid composition were used, it became evident that only the amount of DPPC in the film just before the compression determines the γ_{min} value of the compressed film and not the abundance of SP-C. This is illustrated by the following experiment (Fig. 3): an 80% DPPC film containing SP-C was cycled with 0% DPPC (80% POPC) vesicles in the subphase. When new lipids are inserted into the bubble interface during the first expansion, the 80% DPPC surface film will be diluted with POPC. The surface area is increased almost twofold in our experimental setup and, when SP-C is present, the surface tension does not change much during the first expansion of the bubble. This means that the amount of lipids at the interface is increased twofold, and a resulting final DPPC content of 40 to 50% is expected. The first compression of the bubble led to high γ_{min} values of approximately 20 mN/m, comparable to, or even higher than the γ_{min} values in the experiment where both the subphase and the film consisted of 50% DPPC (Fig. 2). The dilution effect was also observed when

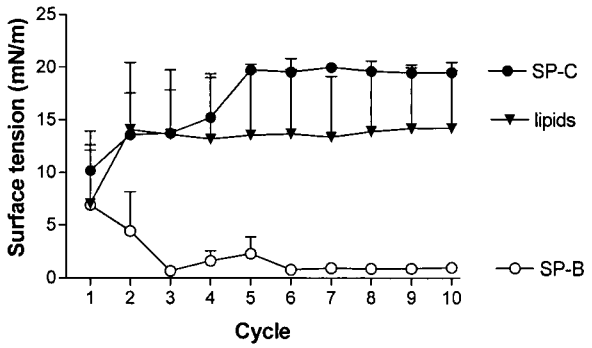

FIGURE 2 Minimum surface tensions of 50% DPPC films upon cycling. Surface films containing 50% DPPC were spread with or without proteins. Subphase vesicles consisted of 50% DPPC without proteins (1 μmol PL/ml). Values shown are the averages of at least three separate experiments ± SEM.

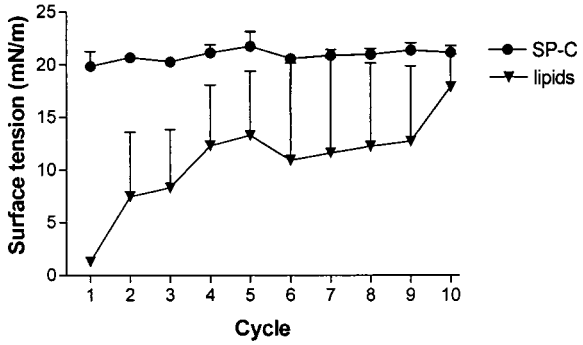

FIGURE 3 Minimum surface tensions of 80% DPPC films upon cycling with 0% DPPC vesicles present in the subphase. Surface films were spread containing 80% DPPC with or without proteins. Subphase vesicles consisted of 0% DPPC (1 μmol PL/ml) without proteins. Values shown are the averages of at least three separate experiments ± SEM.

no protein was present in film or subphase (Fig. 3). The only difference was that the effect was faster for SP-C-containing films because more lipids were adsorbed from the subphase during expansion of the bubble. That more lipids are adsorbed with SP-C is obvious from the lower γ_{max} values for SP-C-containing surface films compared to protein-free films (not shown, but comparable to the other lipid mixtures in Table 1). When SP-B was incorporated in the film, it was able to create a γ_{min} of 4 mN/m upon the first compression (not shown). However, the dilution effect was only counteracted by SP-B in the first cycle and further cycling led to γ_{min} values of 20 mN/m. The reverse process of feeding a film containing a low amount of DPPC with vesicles rich in DPPC worked as well. A 50% DPPC film containing SP-C became enriched in DPPC when 80% DPPC vesicles were present in the subphase, leading to decreased γ_{min} upon cycling (not shown).

To investigate the role of SP-A in DPPC enrichment of the monolayer, both adsorbed films and spread films were used. Films adsorbed from 40% DPPC vesicles containing both SP-A and SP-B had significantly lower minimum surface tensions than adsorbed films from vesicles containing only SP-B (Fig. 4). The samples had comparable fast adsorption kinetics and both films reached the equilibrium surface tension of 22 mN/m within 1 min. Films adsorbed from vesicles that contained only SP-A failed to reach the equilibrium surface tension after 15 min adsorption (not shown). Consequently, the surface activity during cycling was low. To study the role of SP-A further, spread films containing 40% DPPC and SP-B were cycled with subphase vesicles containing 40% DPPC, and SP-A or SP-B or both proteins. The results are depicted in Fig. 5. From the γ_{min} values it can be seen that only when both proteins were present in the subphase vesicles, a DPPC enrichment is observed upon every compression. However, the effect on DPPC enrichment was slower compared to adsorbed films (compare Fig. 4 and Fig. 5). When only SP-A was present

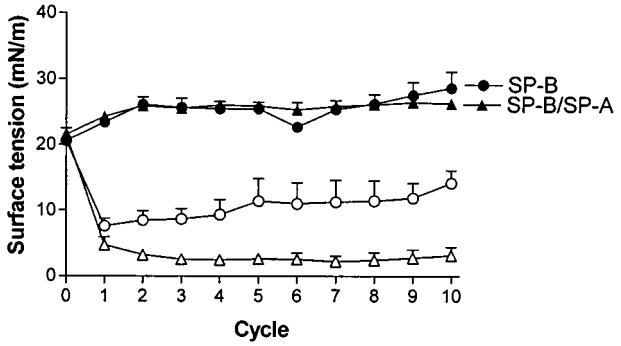

FIGURE 4 Effect of SP-A on surface film refinement using adsorbed films. Surface films were adsorbed from 40% DPPC subphase vesicles (1 μmol PL/ml) and subsequently cycled. Open symbols represent γ_{min} values; closed symbols represent γ_{max} values. Values shown are the averages of at least three separate experiments ± SEM.

in the subphase vesicles, no significant differences were observed in the minimum surface tension compared to SP-B-containing subphase vesicles, but a clear negative effect was observed on the maximum surface tensions (Fig. 5). In additional experiments, SP-A was not added to the SP-B-containing vesicles, but injected directly into the sample chamber, followed 5 min later by injection of the vesicles. In these experiments, no effect of SP-A was observed (not shown).

Similar experiments were conducted using combinations of SP-A and SP-C. In spread films of 50% DPPC and SP-C higher γ_{min} and γ_{max} were measured during cycling when SP-A was present in the subphase than when it was absent. The same effect of SP-A was observed using adsorbed films: adsorption from subphase vesicles was slower in the presence of SP-A and inferior surface activity was observed during cycling (not shown). This suggests that the DPPC

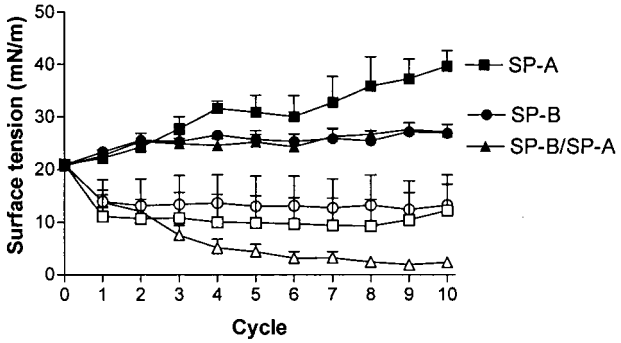

FIGURE 5 Effect of SP-A on surface film refinement using spread films. Surface films were spread containing 40% DPPC and SP-B. Subphase vesicles (1 μmol PL/ml) consisted of 40% DPPC with SP-A and/or SP-B. Open symbols represent γ_{min} values; closed symbols represent γ_{max} values. Values shown are the averages of at least three separate experiments ± SEM.

enriching effect of SP-A occurs only in combination with SP-B.

DISCUSSION

In this study we tested the roles of surfactant proteins A, B, and C in refinement of surface films. In most model systems used to test the surface activity of surfactant proteins, a standard lipid mixture containing 70–80% DPPC is used. An increased surface activity due to a lowered proportion of DPPC was observed by Holm et al. (1996), but we did not detect such an increase. We lowered the DPPC content down to limiting conditions leading to increased minimum surface tensions during cycling of the air bubble. Subsequently, we determined to what extent the surfactant proteins were capable of enriching the surface film at the interface in DPPC.

Using spread films of 50% DPPC, it was clear that SP-B was capable of lowering the γ_{min} values to near zero during the cycling procedure (Fig. 2). These low surface tensions can be achieved only when the interface is completely covered with DPPC. This implies that the decreasing γ_{min} values are correlated to enrichment in DPPC of the interface. SP-C did not show this behavior at all. This is in contradiction with some studies where a role for SP-C in this respect was proposed (Wang et al., 1996a,b; Venkitaraman et al., 1991; Qanbar et al., 1996), but observations similar to ours in pulsating bubble surfactometer experiments have been described as well (Yu and Possmayer, 1990). Quite remarkable is also the fact that during the cycling procedure increasing γ_{min} and γ_{max} were observed with SP-C in several lipid compositions (Table 1 and Fig. 2). One of the major roles of SP-C is thought to be the creation of a lipid reservoir that can accommodate lipids squeezed out of the interface during compression. These lipids can re-enter upon the next expansion of the bubble or, in vivo, the alveolus. This lipid reservoir formation activity of SP-C was recently visualized using scanning force microscopy (von Nahmen et al., 1997b) on films formed on a Wilhelmy surface balance. However, in our experimental setup, material was quite clearly lost from the interface. Whether this is due to the dynamic system that we used or to the lipid composition is unclear.

Enrichment of the monolayer in DPPC can occur in two ways: selective adsorption of DPPC or selective removal of non-DPPC lipids. In captive bubble experiments, it has been observed that SP-A has a positive effect on specific DPPC adsorption (Schürch et al., 1992). In those experiments films were formed by adsorption. Low amounts of lipid extract surfactant (containing SP-B and SP-C) were used in the subphase as the limiting factor. When SP-A was added to the mixture, the film required less area reduction to reach a near-zero surface tension. This implies that less non-DPPC lipids have to be removed from the interface upon compression, or, in other words, that a higher amount of

DPPC is already present in the interface. In our experiments we saw a similar effect for simple lipid mixtures containing SP-B and SP-A. The DPPC enrichment, reflected in decreasing γ_{min} values, was faster when adsorbed films were used than with spread films: a γ_{min} of approximately 4 mN/m was reached after the second cycle in adsorbed films (Fig. 4), whereas 6 cycles were required with spread film to achieve this surface tension (Fig. 5). This indicates that SP-A, combined with SP-B enhances specific DPPC adsorption. In the adsorbed film specific DPPC adsorption has to a large extent occurred during formation of the film starting from a clean interface of the bubble. In spread films, DPPC-specific adsorption can only occur during cycling; hence, the effect is smaller.

The effect of SP-A seems to be specific for the combination with SP-B. When SP-C was present, a negative effect was observed on adsorption when SP-A was added to the vesicles. This can be explained by the fact that SP-A will have a lipid aggregating effect on the subphase vesicles in the calcium-containing buffer that we used (Haagsman et al., 1991). This effect is easily detected by eye. The aggregated structure of the vesicles will likely hamper surface film formation, as has been observed before in our laboratory (unpublished results). A similar SP-A-induced lipid aggregation was seen when SP-B-containing vesicles were used and was even increased by the vesicle fusing activity of SP-B. However, the large aggregates that were formed in the presence of SP-B are apparently structurally different from the ones formed in the presence of SP-C, because they seem able to induce specific DPPC adsorption, which is reflected in the lowered γ_{min} during cycling (Fig. 4). We suggest that in these structures DPPC-enriched domains are formed under the influence of SP-A and SP-B, and that these domains are then inserted into the monolayer. When SP-A was injected into the subphase before vesicle injection, no effect was observed. The calcium-containing buffer in the sample chamber will cause self-aggregation of SP-A. Apparently this self-aggregation hampers the DPPC domain formation of the subphase vesicles, and thereby the positive effect of SP-A on the surface activity. The possible role of SP-A in the process of DPPC domain formation is supported by several observations: SP-A specifically binds DPPC (Kuroki and Akino, 1991) and interacts with the boundaries between condensed and fluid regions in DPPC monolayers (Ruano et al., 1998). Besides that, SP-A was shown to aggregate DPPC molecules in DPPC/cholesterol mixtures in vitro (Yu and Possmayer, 1998). Although we have no structural data on the formed structures, they could even have resemblance to tubular myelin, since it has been shown that reconstitution of SP-A, SP-B, DPPC, and PG can lead to tubular myelin formation in the presence of calcium ions (Suzuki et al., 1989). In addition, Venkitaraman et al. (1990) showed that SP-A combined with SP-B counteracted the inhibitory effect of albumin and hemoglobin on the surface activity of simple lipid mixtures This effect was not observed for combinations of SP-A and SP-C, which the authors also attributed to the formation of the specific large aggregated lipid structures by SP-A and SP-B.

Comparison of adsorbed films of SP-B in 40% DPPC with spread films of the same composition (Figs. 4 and 5) shows that comparable γ_{min} values are achieved on the first compression. This indicates, contrary to some recent captive bubble studies (Schürch et al., 1998; Nag et al., 1999), that SP-B alone does not induce specific DPPC adsorption, because a lower γ_{min} would then be expected for adsorbed films (as observed for SP-A/SP-B mixtures, Figs. 4 and 5). This indicates that SP-B enriches a surface film in DPPC by specific removal of non-DPPC lipids during cycling.

Another interesting feature is observed when limiting amounts of 40–50% DPPC are used in surface films. Often, either a very low γ_{min} of 2 mN/m or a high γ_{min} of approximately 20 mN/m is reached upon compression of the bubble (all-or-nothing). One of the characteristics of SP-B, and in some experiments also SP-C, is that these proteins can stabilize films with these minimum surface tensions between 2 and 20 mN/m. In contrast, films that contain only phospholipids do behave according to the all-or-nothing principle. For example, in Fig. 3 a rising γ_{min} is observed with an average value of 13 mN/m at cycle 5. This average value is built up from experiments where some samples reached a γ_{min} of approximately 2 mN/m, and some experiments had a γ_{min} of 20 mN/m. A value of 13 mN/m is, in our experimental setup, almost never observed in a single separate experiment with protein-free phospholipid films.

Recently an interesting finding was published by Crane and Hall (2000). They showed that the speed of compression of the air bubble in the CBS has an effect on the minimum surface tension that is reached. Fast compressions of a film formed from calf surfactant in a CBS led to low minimum surface tensions, whereas a slow compression resulted in γ_{min} values >20 mN/m. Similar results were observed for dimyristoylphosphatidylcholine monolayers. These findings indicate that compression rates must be standardized and care must be taken when conclusions are drawn from γ_{min} values in CBS studies.

In summary, we used simple lipid mixtures of DPPC, POPC, and POPG to determine the role of surfactant proteins in refining a surface film. The combination of spread films and adsorbed films enabled us to distinguish between two mechanisms of DPPC enrichment of the surface film. SP-B enhanced the removal of non-DPPC lipids during cycling, whereas SP-A, when combined with SP-B in vesicles, stimulated the DPPC-specific adsorption. No effect in refinement of the surface film was observed for SP-C.

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