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ARTICLE

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Effects of pulmonary surfactant proteins SP-B and SP-C and calcium ions on the surface properties of hydrophobic fractions of lung surfactant

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Abstract This study focused on two hydrophobic fractions (HF-A and HF-B) isolated from porcine lung surfactant (LS) that had similar phospholipid composition, but HF-A consisted of the hydrophobic LS specific proteins (SP-B and SP-C), in contrast to HF-B. Monolayers spread in a Langmuir trough were formed at the air/water interface of both fractions and the rate of adsorption-desorption and the respreading potential of the LS constituents was studied during six consecutive compression/decompression cycles of the monolayers. By drawing a comparison between the behavior of HF-A and HF-B monolayers on the subphase of 150 mM NaCl, either with or without additional Ca^{2+}, we estimated the role of hydrophobic LS proteins and Ca^{2+} ions for LS surface activity. The results demonstrated much higher ability of the HF-A sample, compared to HF-B, to maintain lower surface tension (γ) during monolayer compression and its better respreading capacity during decompression. For instance, at a surface concentration corresponding to 80 $Å^2$ per phospholipid molecule, the HF-A monolayers showed a much lower γ_{max} value (surface tension at 100% of the trough area), being ca. 31.0 mN/m, compared to the HF-B monolayers ($\gamma_{max} \cong 62.0$ mN/m). The surface tension after compression to 20% of the initial area (γ_{min}) reached ca. 7.0 and 19.0 mN/m in the HF-A and HF-B monolayers, respectively. Better respreading of the HF-A monolayers compared to the HF-B monolayers was due to the faster adsorption and spreading of LS phospholipids during decompression, facilitated by the hydrophobic proteins. As the phospholipid composition of both fractions was similar, we showed that the hydrophobic surfactant proteins were responsible also for the prevention of the irreversible loss of material from the surface during monolayer compression/decompression. The effects observed demonstrated also that the hydrophobic surfactant proteins were the stronger determinant, compared with Ca^{2+} ions, for the surface tension decrease and respreading of the monolayers during film compression/decompression. For instance, when the HF-A monolayers were spread on a subphase with an additional 5 mM Ca^{2+} ion content, no significant changes were detected in the γ_{min} and γ_{max} values between the first and sixth cycle, compared to the monolayers spread on a subphase of 150 mM NaCl only. However, in the absence of positively charged SP-B and SP-C (HF-B sample) in highly compressed monolayers, Ca^{2+} ions were able to cause the effects shown by SP-B and SP-C, although to a less extent. The role of the electrostatic and hydrophobic interactions is discussed for the better respreading of LS components in the presence of LS proteins and Ca^{2+} ions.

Key words Lung surfactant · Surfactant proteins · Lipid-protein interaction · Calcium ions · Monolayers

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Introduction

Lung surfactant (LS) is a unique mixture of phospholipids and proteins, found at the air/water interface in the alveoli, whose main function is to lower surface tension during expiration and thus to stabilize the alveoli (Pattle 1955; Clements 1957). Failure to reduce the surface tension results in life threatening respiratory complications in neonates and adults. The major surface lowering agent is dipalmitoylphosphatidylcholine (DPPC), but other phospholipids like phosphatidylglycerol (PG) and minor phospholipid constituents are also very important for maintaining the alveolar stability (Notter 1984; van Golde et al. 1988; Wang et al. 1995). Lung surfactant contains four surfactant-specific proteins: SP-A, SP-B, SP-C, and SP-D, which are involved in the modulation of surface activity of the lipid components and/or the immune defense of the lung (Johansson et al. 1994; Johansson and Curstedt 1997). The hydrophobic surfactant-specific proteins SP-B and SP-C are considered to play an essential role in the adsorption of phospholipid constituents of LS at the air/water interface, and in the desorption and respreading during alveo-

lar dynamics, etc. (Curstedt et al. 1987; Yu and Possmayer 1990). The exact mechanism of the effect of the interactions between phospholipids and SP-B and SP-C is still not known. Extensive studies have been carried out on these effects and the results have been applied in the clinical treatment of surfactant disorders with exogenous surfactant preparations, some of which consist of SP-B and SP-C (Hall et al. 1992). SP-B and SP-C could exist in hydrophobic fractions isolated from animal LS, depending on the extraction procedure, and thus their importance for biophysical activity in LS could be studied (Whitsett et al. 1986).

Calcium ions are involved at different stages of surfactant conversion and function, for example in tubular myelin formation and in acceleration of the adsorption of phospholipids to the air/water interface (Hawgood et al. 1987; Suzuki et al. 1989). Different mechanisms of Ca^{2+} effects on the LS surface activity have been proposed with a view to modifying the lipid-protein interactions and electrostatic forces among surfactant components (Davies et al. 1986; Oosterlaken-Dijksterhuis et al. 1991; Yu and Possmayer 1992). Both in vivo and in vitro model studies have been applied for investigating the role of calcium ions in the molecular interactions in LS.

Most of the model studies on the interactions between LS phospholipids and proteins have used pure DPPC and PG and purified (or recombinant) SP-B and SP-C and, in general, the role of minor lipid constituents has not been taken into account. In addition, the concentrations of SP-B and SP-C used in these studies are usually much higher (up to 20 wt%) than those observed in vivo (Taneva and Keough 1994a, b, c). In other studies, hydrophobic fractions, isolated from native LS of animal lungs with protein content as in vivo, but lacking some single constituents (phospholipids, neutral lipids, etc.), have been investigated (Hall et al. 1994; Wang et al. 1995, 1996; Lalchev et al. 1996).

In the present study, two hydrophobic fractions (HF) of porcine LS with similar phospholipid composition were isolated. One of the fractions consists of SP-B and SP-C in concentrations similar to those in native LS, but the other one lacks the hydrophobic proteins. Thus, by means of the comparative study of the two fractions we estimated the role of SP-B and SP-C in in vivo concentrations for the surface activity and monolayer behavior of LS. With a view to studying the adsorption-desorption rate and respreading potential of the LS constituents, spread monolayers at the air/water interface (the same as in the alveoli) of both fractions were formed in a Langmuir trough and their dynamic behavior was studied during consecutive compression/decompression cycles. Another aim of this study was to estimate the role of calcium ions both in the presence and absence of SP-B and SP-C for the dynamic characteristics of the monolayers of the hydrophobic fractions.

Materials and methods

Isolation of hydrophobic fractions of lung surfactant

Lung surface active material (LSAM) was obtained by washing porcine lungs with 0.14 M NaCl. The lavage fluid was subjected to a series of centrifugations according to Ng et al. (1983). The final purification was performed by sucrose gradient centrifugation (0.68 M and 0.33 M sucrose, 78 000 *g*, 60 min, 4 °C) and the obtained LSAM was used for isolation of LS hydrophobic fractions.

Two hydrophobic fractions (HF-A and HF-B) of LS were isolated by extraction using different solvent systems. In order to obtain the HF-A fraction the resuspended LSAM was extracted by chloroform/methanol using the method of Bligh and Dyer (1959). The lower chloroform layer was evaporated under a stream of nitrogen and then dissolved in 0.5 ml chloroform for phospholipid determination and surface activity study. The HF-A fraction consists mainly of a mixture (or complex) of LS phospholipids, neutral lipids, and the hydrophobic LS proteins, SP-B and SP-C. The HF-B fraction was isolated through extraction of LSAM by diisopropyl ether/*n*-butanol under conditions in which SP-B and SP-C remained in the aqueous phase (Cham and Knowels 1976). The upper organic phase was collected and evaporated under a stream of nitrogen and dissolved in 0.5 ml chloroform. The HF-B fraction consists of LS lipids only.

Composition of HF-A and HF-B

Individual phospholipids were separated by thin layer chromatography using silica gel G plates (Merck) and solvent system B as described by Touchstone et al. (1980). The content of both the total and individual phospholipids was determined by inorganic phosphorus assay after mineralization of the samples (Kahovkova and Odavic 1969).

In order to determine the protein content of each fraction, we used amino acid analysis because the extremely high relative content of lipids in the samples compromises the determination of proteins by conventional methods. In determining the individual amino acids the Ser content was ignored, because of its possible origin from phosphatidylserine resulting in a high error of determination. The Cys content was determined separately after oxidation of the sample.

Surface activity study

Spread monolayers were formed in a Langmuir trough with dimensions 95×18 mm (Biegler Electronic, Austria) and a monolayer area equal to 1710 mm^2. Surface tension measurements were carried out through the Wilhelmy plate method. A certain amount of phospholipids in chloroform was spread at an initial surface concentration of 80 $Å^2$/molecule on the subphase containing 150 mM NaCl in

doubly distilled water. In the experiments related to studying the role of calcium ions on monolayer behavior, the subphase contained 150 mM NaCl plus 5 mM $CaCl_2$. The surface tension γ(mN/m) during compression/decompression between 100% (γ_{max}) and 20% of the initial monolayer area (γ_{min}) was monitored. The compression/decompression rate was 1.5 min per cycle. The monolayer surface tension was monitored up to the sixth cycle in our studies. Each experiment was repeated at least three times ($n \geq 3$). The temperature in all experiments was 22±1 °C.

Results

The phospholipid and protein composition of the hydrophobic fractions HF-A and HF-B are shown in Tables 1 and 2. The two fractions showed almost identical phospholipid profiles typical for LS, with phosphatidylcholine being the predominant component (74–76 wt%), followed by phosphatidylglycerol (7–8 wt%). The two fractions differ mainly in the negatively charged phosphatidylinositol (PI) and phosphatidic acid (PA), which are present in HF-A (3.2 wt%) but not in HF-B (Table 1). The absence of PI + PA in the HF-B fraction can be connected with their strong interaction with positively charged SP-B and SP-C (Perez-Gil et al. 1995), resulting possibly in a complex formation which moves to the aqueous phase during the extraction procedure and thus is absent in the organic phase (HF-B fraction). Differences in the solubility of different phospholipid species in chloroform/methanol or diisopropyl ether/*n*-butanol (see Materials and methods) could also be in the origin of the compositional differences between HF-A and HF-B (Table 1).

Amino acid analysis of the fractions indicated HF-A amino acid composition (Table 2) typical for SP-B and SP-C (Curstedt et al. 1988; Johansson et al. 1988; Christova et al. 1996) and the absence of hydrophobic proteins in HF-B (Bates et al. 1992). SP-B and SP-C are known as extremely hydrophobic proteins due to their unique high content of hydrophobic amino acids. In Table 2 is shown the sum of the molar% of the hydrophobic amino acids in HF-A (77.8 mol%), which compares favorably with other published data for SP-B (74.3%) (Curstedt et al. 1988) and SP-C (89.5%) (Johansson et al. 1988). The comparison of the mol% of each amino acid in HF-A was in good accordance with those in the hydrophobic proteins SP-B and SP-C from the literature (data not shown) except for Val, whose content was strongly underestimated in our study probably due to the incomplete hydrolysis of six consecutive Val groups in SP-C (Johansson et al. 1988). The HF-A protein quantification based on our amino acid analysis revealed that HF-A contained 0.84±0.03 wt% of SP-B plus SP-C. The lower, according to the literature, content of proteins obtained by us is due to the incomplete hydrolysis of the sample, the strong underestimation of Val, and the exclusion of Ser in our study.

The results presented in Tables 1 and 2 show that the isolated hydrophobic fractions possess phospholipid composition typical for LS and differ in their SP-B + SP-C and PI + PA content, all being present in HF-A but not in HF-B. In the limits of our results, however, we are not able to prove whether positively charged proteins and negative phospholipids present in the HF-A sample are formed or not in the complexes.

Representative surface tension/area isotherms during film compression/decompression cycles for both fractions are shown in Fig. 1. This illustrates the occurrence of a significant difference between the shape of the curves of the HF-A and HF-B fractions at equal initial surface concentrations of phospholipids (corresponding to 80 $Å^2$/molecule). Much lower γ_{max} (surface tension at 100% area of the film) and γ_{min} (surface tension after compression to 20% of the initial area) values were detected for HF-A monolayers, compared to those of HF-B. During compression of the films, the HF-A monolayer reached its lowest surface tension values much earlier (ca. at 40% area) than the HF-B monolayer, which reached its lowest γ at the end-compression point. During decompression, a plateau was observed between 35% and 65% of the initial area for HF-A monolayers. In contrast, the surface tension in-

Table 1 Phospholipid composition of HF-A and HF-B samples

Phospholipid	HF-A (wt%)	HF-B (wt%)
Sphingomyelin	2.2±0.2	2.4±0.2
Phosphatidylcholine	74.3±3.2	76.4±3.1
Phosphatidylserine	4.2±0.2	4.6±0.3
Phosphatidylinositol + phosphatidic acid	3.2±0.3	0
Phosphatidylethanolamine	5.4±0.3	6.9±0.4
Phosphatidylglycerol	8.2±0.4	7.3±0.5
Cardiolipin	2.5±0.3	2.4±0.4

Table 2 Amino acid composition of HF-A sample

Amino acid	HF-A (mol%)
Phe	1.03
Leu	21.31
Ile	5.09
Tyr	0.54
Trp	n.d.[c]
Val	11.92
Met	2.50
Pro	11.42
Cys	10.32
Ala	4.53
Gly	9.13
Thr	3.30
Ser	[b]
Lys	4.73
His	0
Glx	2.65
Asx	3.06
Arg	8.22
∑ Hydrophobic amino acids[a]	77.8%

[a] According to Black and Mould (1991)
[b] The content of Ser was ignored because of the possible origin from phosphatidylserine
[c] n.d. = not determined

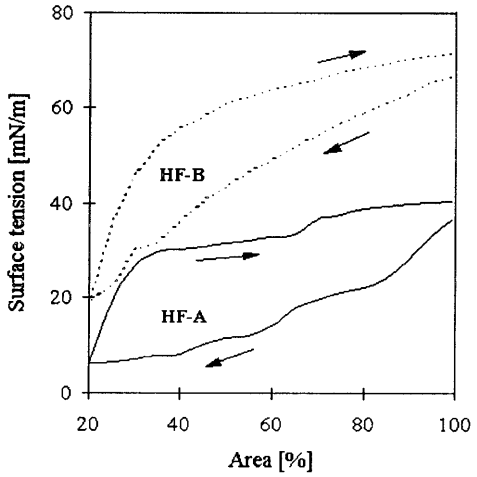

Fig. 1 Surface tension-area isotherms of HF-A and HF-B monolayer films. HF-A and HF-B were spread from chloroform solution on the subphase, containing 150 mM NaCl. The surface tension was monitored by the Wilhelmy plate method. Rate of compression/decompression = 1.5 min/cycle. Temperature = 22±1°C. Representative curves of the third cycles of compression/decompression for both samples are shown ($n \geq 3$). ← Compression of the monolayers; → decompression of the monolayers

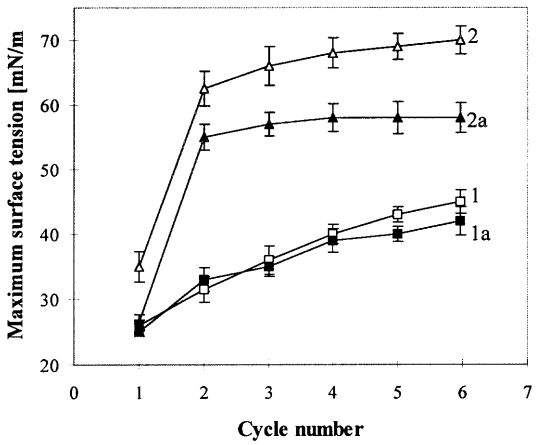

Fig. 2 Dependence of γ_{max} (surface tension at 100% of the trough area) of HF-A and HF-B spread monolayers on the cycle number. HF-A and HF-B were spread from chloroform solution on the subphase, containing 150 mM NaCl (curves 1 and 2, respectively) or 150 mM NaCl plus 5 mM $CaCl_2$ (curves 1a and 2a, respectively). The surface tension was measured by the Wilhelmy plate method during six consecutive cycles of film compression/decompression between 100% and 20% of the initial area. Rate of compression/decompression = 1.5 min/cycle. Temperature = 22±1°C. Data presented are averaged from at least three experiments ($n \geq 3$). □ and ■, HF-A fraction; △ and ▲, HF-B fraction

creased gradually during decompression when the HF-B monolayers were expanded, reaching a much higher γ value at 100% area (ca. 70 mN/m) than the HF-A monolayers (ca. 39 mN/m). These results illustrate (1) the much higher capacity of the HF-A sample to lower the surface tension during film compression and (2) the much better respreading of HF-A films during decompression, compared to the HF-B films.

Figure 2 illustrates the dependence of γ_{max} values on the cycle number during six consecutive compression/decompressions cycles of the monolayers obtained from the two hydrophobic fractions. The results indicate the occurrence of much higher γ_{max} values after each cycle for monolayers obtained from HF-B (curve 2) compared to HF-A (curve 1). For both curves it was observed that the γ difference was most significant in the range of the first two cycles. The difference of γ_{max} values between the first and second cycles was 5 mN/m for HF-A and 28 mN/m for HF-B films. These results showed much better respreading during decompression of the films of HF-A compared to those of HF-B. The curves of the increase of γ_{max} during the cycle numbers obviously reflect the respreading potential of the samples studied, but also the irreversible loss of molecules from the surface owing to their irreversible desorption during compressions. It becomes obvious from Fig. 2 that the total loss of material from the surface after the sixth cycle is significantly lower for the films of HF-A (γ_{max} = 42.0 mN/m) than for HF-B (γ_{max} = 70.0 mN/m).

The presence of Ca^{2+} ions in the subphase showed different effects on the change of γ_{max} values during cycling of the monolayers formed from both fractions (curves 1a and 2a in Fig. 2). The surface tension of the HF-A sample did not change significantly when the subphase contained 5 mM Ca^{2+} ions, compared with the case when the subphase was depleted of Ca^{2+}. In contrast, a significant decrease of γ_{max} values for the HF-B films was observed after the addition of Ca^{2+} ions to the subphase (compare curves 2 and 2a in Fig. 2). The effect of the γ_{max} decrease from the monolayers of the HF-B sample, being approximately 8–12 mN/m depending on the cycle number, remained almost the same with the increase of Ca^{2+} concentration up to 30 mM (data not shown). The results in Fig. 2 illustrate that the effect of the γ_{max} decrease is boosted to a greater extent by the addition of hydrophobic proteins to the pure phospholipid films (compare curve 2 and curve 1) than the same effect observed after addition of 5 mM Ca^{2+} ions (compare curve 2 with curve 2a). The latter observation illustrates that the hydrophobic surfactant proteins (being positively charged) are the stronger determinant, compared to Ca^{2+} ions, for maintaining the low γ_{max} values during film compression/decompression. Therefore not only electrostatic interactions but also hydrophobic ones contributed to the effects thus observed. We can also conclude that, as regards the γ_{max}, with SP-B and SP-C in the monolayers the effect of Ca^{2+} ions could be ignored (compare curves 1 and 1a).

The dependence of γ_{min} of the monolayers formed from both fractions on the cycle number is illustrated in Fig. 3, namely that a better HF-A surface activity is detected by the change of γ_{min} values after comparing consecutive HF-A and HF-B monolayer compression/decompressions (curves 1 and 2, respectively). Using LS monolayer studies, many authors consider that γ_{min} values of less than 10 mN/m are needed to maintain the alveolar stability during expiration (Clements et al. 1958, 1961; Clements and

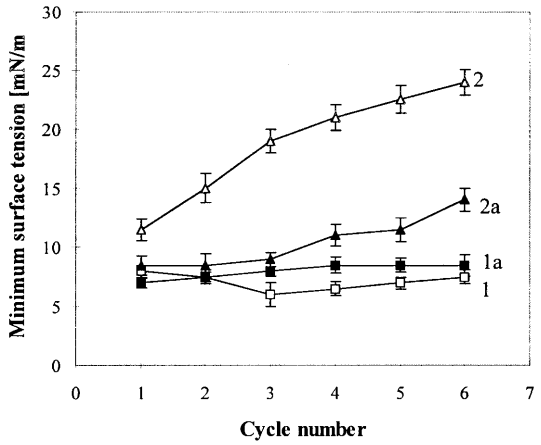

Fig. 3 Dependence of γ_{min} (surface tension at 20% of the trough area) of HF-A and HF-B spread monolayers on the cycle number. HF-A and HF-B were spread from chloroform solution on the subphase, containing 150 mM NaCl (curves 1 and 2, respectively) or 150 mM NaCl plus 5 mM $CaCl_2$ (curves 1a and 2a, respectively). The surface tension was measured by the Wilhelmy plate method during six consecutive cycles of film compression/decompression between 100% and 20% of the initial area. Rate of compression/decompression = 1.5 min/cycle. Temperature = 22±1°C. Data presented are averaged from at least three experiments ($n \geq 3$). □ and ■, HF-A fraction; △ and ▲, HF-B fraction

Tierney 1965; Schurch et al. 1976). γ_{min} values for the HF-A monolayers of between 5 and 8 mN/m were detected between the first and the sixth cycles, starting with a slight decrease up to the third cycle and followed by a continuous recovery of the initial value between the fourth and sixth cycles (curve 1 in Fig. 3). The effect thus observed up to the third cycle could be linked with the possible "purifying" of the monolayer surface from some unsaturated phospholipids (or lipid-protein complex) compressed to such a low value of γ (van Golde et al. 1988; Bangham et al. 1979). In contrast, much higher γ_{min} values of the monolayers were measured for the HF-B samples (curve 2 in Fig. 3). Obviously the γ_{min} values gradually increased during cycling, resulting in a difference of approximately 12 mN/m between the first and the last cycle. Following the monolayer spreading on a subphase containing Ca^{2+} ions, no significant changes were detected in the γ_{min} values between the first and sixth cycles for HF-A monolayers (curve 1a in Fig. 3). No change in γ_{min} values in the presence of Ca^{2+} were observed when a HF-A sample was spread at the initial concentration of 150 $Å^2$/molecule (data not shown). In contrast, in the presence of Ca^{2+} ions the HF-B monolayers reached much lower γ_{min} values than those in the absence of Ca^{2+} (compare curves 2 and 2a). It was interesting to notice that, up to the third cycle, Ca^{2+} ions were equally efficient as SP-B and SP-C in maintaining the low values of γ_{min}. The latter observation could be connected with the possible binding of Ca^{2+} ions with the phospholipids in the conditions of high monolayer density (after compression to 20% of the area), resulting in an increased surface packing of the phospholipids, and hence low γ_{min}. A phase separation, induced by Ca^{2+} ions (Ito and Ohnishi 1974; Silvius and Gagne 1984), could also facilitate the squeeze-out of unsaturated phospholipids during film compression, and thus to contribute to maintaining low γ_{min} values.

Discussion

There are several approaches in the literature to studying through monolayers the role of LS constituents in maintaining alveolar stability by lowering the monolayer surface tension. Some authors study separately the surface behavior of the pure LS phospholipids (mainly DPPC and PG) (e.g. Notter et al. 1980) or pure surfactant proteins (e.g. Taneva and Keough 1994a, b, c). Another approach uses mixed monolayer films of phospholipids (DPPC and PG) and hydrophobic surfactant proteins (Taneva and Keough 1994a, b, c, d, 1995; Yu and Possmayer 1992) and allows study of the interactions between the LS components and the impact of SP-B and/or SP-C on monolayer behavior. However, the use of synthetic phospholipids in the above-mentioned studies was unfavorably compounded by the use of much higher concentrations of SP-B and SP-C (up to 20 wt%) than those observed in vivo (1–3 wt%), although the real protein content at the air/water interface of the lungs might be much higher. More realistically in other cases, several natural LS fractions containing different hydrophobic LS components (phospholipids, neutral lipids, and hydrophobic surfactant proteins) were isolated from animal lungs (e.g. by Sephadex LH-20 chromatography, Hall et al. 1994), and a comparison was made of their surface activities on the monolayers (Wang et al. 1995, 1996). This approach using natural samples with different compositions could provide more precise information of how a single-fraction component reflects the LS ability to lower the surface tension and its respreading capacity, although one should consider that monolayers spread from organic solvents may not be the same as in the alveoli.

In our study we have isolated two natural hydrophobic LS fractions (HF-A and HF-B) by extraction through different solvent systems, making it possible to obtain fractions with similar phospholipid composition, but only one (HF-A) contained both the typical (for LS) hydrophobic SP-B and SP-C in concentrations similar (or lower) to those observed in vivo and the negatively charged phospholipids PI and PA. By comparison between the behavior of HF-A and HF-B monolayers on a subphase containing or not containing Ca^{2+}, we estimated the role of the hydrophobic LS proteins and Ca^{2+} ions on LS surface activity.

The surface tension-area isotherms of the two fractions showed significant differences during the monolayer compression and expansion not only in γ_{max} and γ_{min} values, but also in the entire shape of the isotherms. The HF-A monolayers reached the lowest surface tension values much earlier during compression, compared to HF-B monolayers (Fig. 2). The differences are caused by the presence of SP-B and SP-C (and charged PI and PA) in the HF-A fraction, where more soluble (than pure DPPC) lipid-

protein complexes may be formed. The formation of complexes ready for desorption during compression could result in the enrichment of the monolayer in the highly surface active DPPC molecules and thus facilitate the achievement of low γ. The better respreading of HF-A monolayers compared to HF-B monolayers illustrated by the plateau between 35% and 65% in the decompression curve could be interpreted (1) as a faster adsorption and spreading of LS phospholipids during decompression facilitated by the hydrophobic proteins and (2) as a rapid reversible respreading at the air/water interface of the possible formed lipid-protein complexes during compression/decompression cycling.

In monitoring the γ_{max} values, a great increase of 28 mN/m was observed during the first and the second cycles of the HF-B monolayers, showing the irreversible loss of material in the absence of SP-B and SP-C. In contrast, in the HF-A monolayers the irreversible loss of material is reduced, resulting in the difference in γ_{max} of 5 mN/m between the first and the second cycles (Fig. 3). In addition, the HF-A monolayer maintains almost constant γ_{min} values, in contrast to the HF-B monolayer, during the six consecutive compression/decompression cycles (Fig. 3). These results confirm some data in the literature indicating that the presence of surfactant proteins improves the respreading of DPPC on continuous cycling in the collapse regime (Taneva and Keough 1994d; Wang et al. 1995).

In many cases, calcium ions have proved to play a substantial role in the physiological activity of LS (Davies et al. 1986; Hawgood et al. 1987; Oosterlaken-Dijksterhuis et al. 1991; Yu and Possmayer 1992), such as influencing the tubular myelin formation, accelerating the phospholipid adsorption to the air/water interface, etc. (Hawgood et al. 1987; Suzuki et al. 1989). There are several model studies on the effects of calcium ions on the properties of spread monolayers of pure phospholipids and hydrophobic surfactant proteins in dynamic conditions (e.g. Yu and Possmayer 1992; Taneva and Keough 1995). Ca^{2+} ions have been shown to influence the interaction between SP-B/SP-C and dipalmitoylphosphatidylglycerol (DPPG) by reduction of the electrostatic forces after formation of neutral DPPG-Ca^{2+} species. Calcium also affects the hydrophobic interactions between DPPG and proteins through its influence on the molecular packing of hydrocarbon chains (Taneva and Keough 1995). However, these results should be transferred to the in vivo conditions carefully because of the high protein concentrations and the lack in the studied samples of unsaturated, neutral, and other charged phospholipids typical for LS. For coming closer to the in vivo conditions, the importance should be noted of the presence of unsaturated, neutral, and charged lipids in the samples owing to their fluidization effect on DPPC monolayers, thus accelerating below 41 °C its adsorption and spreading at the interface (van Golde et al. 1988), etc. The role of minor LS phospholipids is important as well for thermal transitions in the lipid phase state at interfaces (Nikolova et al. 1994). Using the fluorescence recovery after photobleaching method, it was reported also that charged and unsaturated phospholipids accelerated the molecular mobility in the monolayer plane of lipid bilayers (Lalchev et al. 1994, 1995a, b), which refleced (e.g. by the Marangoni effect) an improvement of the spreading in mixed phospholipid films (Lalchev 1997). Unfortunately, in the limits of this study we were not able to recognize the contribution (if it exists) of the charged PI and PA from that of SP-B and SP-C for the effects observed on HF-A monolayers. Since the PI + PA content is very small in the HF-A lipid pool and the change of other charged phospholipids between HF-A and HF-B is also small (Table 1), we are inclined to address the different effects detected between HF-A and HF-B samples to the presence of SP-B and SP-C in HF-A. Although the content of the hydrophobic proteins in HF-A was not high (ca. 1 wt%), the effects of the γ decrease were well detected in the presence of the proteins (see Figs. 1–3). It should be noted that owing to SP-B/SP-C the much higher molecular weight (and surface area) part of the γ decrease is probably only due to the expansion of the films by the proteins.

In our study we used similar concentrations of SP-B and SP-C as in vivo, or eventually lower owing to the loss of proteins during the extraction procedure. The use of small amounts of hydrophobic proteins in our samples has the advantage that one can compare the effects between the γ decrease in the presence (or absence) of Ca^{2+} and in the presence of such a small protein content. The results showed that where SP-B and SP-C were present (in the HF-A sample), Ca^{2+} ions did not improve significantly the surface activity of the sample, i.e. the hydrophobic surfactant proteins were the stronger determinant compared with Ca^{2+} ions for the surface tension decrease and respreading of the monolayers during film compression/decompression. However, in the absence of SP-B and SP-C (the HF-B sample), Ca^{2+} ions facilitated the reaching of about 30–40% of the effects on γ_{max} caused by SP-B and SP-C (Fig. 2). Moreover, as regards the change of γ_{min} values (i.e. in highly condensed monolayers), the results showed that the effects of Ca^{2+} ions on the behavior of pure phospholipid monolayers were very similar (ca. 80–90%) to those observed for SP-B and SP-C (Fig. 3). These stronger effects of Ca^{2+} ions on the highly condensed monolayers detected in our study could be explained not only by the ability of calcium to bind electrostatically with the phospholipids and/or to induce surface phase separation (Ito and Ohnishi 1974; Silvius and Gagne 1984), but also by its ability to promote dehydration in acidic phospholipids and thus to increase their hydrophobicity (Hauser et al. 1975). The positive charge of Ca^{2+} ions could reduce the repulsive forces (Creuwels et al. 1995) between the monomolecular layer and the possible collapse structures, thus facilitating the rapid adsorption during decompression. Presuming that the hydrophobic SP-B and SP-C are, in addition, positively charged at physiological pH, we can infer that the participation of the electrostatic interactions is of key importance for the better respreading of LS components in the presence of LS proteins and Ca^{2+}. Further work is required, however, to clarify the role of the hydrophobic interactions between LS proteins with phospholipids

(including charged PI and PA) in the presence of Ca^{2+} for the dynamic behavior of monolayers at the air/water interface.

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