Pulmonary Surfactant Protein SP-A with Phospholipids in Spread Monolayers at the Air−Water Interface†

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ABSTRACT: Spread monolayers of pulmonary surfactant protein SP-A, alone or mixed with phospholipid(s), were formed at the air−water interface. Binary monolayers of SP-A plus dipalmitoylphosphatidylcholine (DPPC) showed positive deviations from ideal behavior of the mean areas in the films consistent with partial miscibility and interaction between the protein and lipid. During compression of SP-A/DPPC films which contained ≥5 wt % SP-A, properties were displayed which were consistent with the protein being partially squeezed out at surface pressures of about 30 mN/m. Some protein appeared to remain in the monolayers even when they were compressed to high surface pressures of about 65−70 mN/m, and it was possibly included in the collapse phase(s) that was (were) formed at 72 mN/m. During dynamic cyclic compression−expansion of SP-A/DPPC monolayers initially formed at low surface pressures, SP-A enhanced the respreading of the films compressed beyond collapse compared to the respreading after collapse of films containing DPPC alone. Spread monolayers of SP-A plus either dipalmitoylphosphatidylglycerol (DPPG) or a mixture of DPPC plus DPPG (7:3, mol/mol) displayed additivity of the mean areas in the films, consistent with complete immiscibility (or ideal miscibility, an unlikely effect) between the protein and lipid components. Electrostatic repulsion between SP-A and DPPG, both negatively charged at physiological pH, possibly governed the behavior of these lipid−protein films. Calcium ions in the subphase did not alter the properties of SP-A/DPPC films, whereas they improved the ability of SP-A to mix with DPPG and DPPC/DPPG. Binding of calcium to the negatively charged DPPG and SP-A may account for association of the protein with DPPG and DPPC/DPPG in the monolayers in the presence of the divalent ions.

Surfactant protein A (SP-A)[1] is, by mass, the major pulmonary surfactant-associated protein (Sueishi & Benson, 1981). SP-A is hydrophilic glycoprotein with monomeric molecular weight of 28 000−36 000 (Weaver, 1988; Hawgood & Shiffer, 1991) and isoelectric points ranging from 4.8 to 5.2 (Sueishi & Benson, 1981; Hawgood et al., 1985b), the values varying primarily because of the extent of glycosylation. In aqueous solutions, SP-A is assembled into a complex of 18 monomers with a molecular weight of about 700 000 (Voss et al., 1988; King et al., 1989). The carboxy-terminal region of SP-A has binding sites for carbohydrates (Haagsman et al., 1987) and calcium (Haagsman et al., 1990). Putatively, a region of amphipatic α-helix accounts for the binding of SP-A to phospholipids (Ross et al., 1986).

In vitro studies have shown that SP-A associates with phospholipids that are dispersed in aqueous solutions (King & MacBeth, 1981; King et al., 1983), or immobilized on thin layers of silica gel on plates (Kuroki & Akino, 1991). SP-A caused phospholipid aggregation in a Ca^{2+}-dependent manner (King et al., 1983; Hawgood et al., 1985). In cooperation with the hydrophobic surfactant-associated proteins, SP-A promoted rapid formation of surface films of phospholipids (Hawgood et al., 1987; Chung et al., 1989; Schürch et al., 1992). SP-A was required *in vitro* for generation of tubular myelin, a phospholipid−protein subfraction of surfactant that probably has relevance to the formation of the surface film *in vivo* (Suzuki et al., 1989; Williams et al., 1991). These studies have shown that SP-A is capable of interacting with phospholipid bilayer and multilayer formations, and of modifying their structures and surface activity.

In the present study, we investigated the ability of SP-A to alter surface properties of phospholipids in spread monolayers at the air−water interface. The effect of SP-A on respreading of DPPC after collapse of the surface films was determined. The roles of calcium and the acidic phospholipid DPPG on the interactions of SP-A with lipid monolayers were examined.

EXPERIMENTAL PROCEDURES

Materials. DPPC was purchased from Sigma Chemical Co. (St. Louis, MO) and DPPG from Avanti Polar Lipids Inc. (Alabaster, AL). The lipids were found to be pure by thin-layer chromatography and were used as received. Sodium chloride, calcium chloride, 1-propanol, and amyl

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[1] Abbreviations: cmc, critical micelle concentration; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SP-A, pulmonary surfactant-associated protein; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochoride; X_r, mole fraction of protein amino acid residues in the protein−lipid monolayers determined from the original amounts of spread protein and lipid.

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alcohol, reagent grade, were obtained from Fisher Scientific Co. (Ottawa, ON), and sodium acetate and EDTA from Sigma Chemical Co. were used.

Protein Isolation. Pig lungs were lavaged with 0.15 M NaCl, and the lavage was centrifuged at 800*g* for 10 min. The supernatant was centrifuged at 7000*g* for 60 min. The pellet was resuspended in 5 mM Tris-HCl/100 mM NaCl/1.64 M NaBr (pH 7.4) and centrifuged overnight at 81500*g*. The pellicle was resuspended in 5 mM Tris-HCl/100 mM NaCl (pH 7.4) and centrifuged at 65000*g* for 2 h. SP-A was purified from the surfactant pellet by the method of Haagsman et al. (1987) as follows. The surfactant pellet suspended in water was injected into stirred 1-butanol and then centrifuged at 10000*g* for 20 min. The precipitate was dried under nitrogen and washed twice in 10 mM HEPES/100 mM NaCl/20 mM octyl β-D-glucopyranoside (pH 7.4). Each wash was followed by centrifugation at 100000*g* for 30 min. The material that was insoluble in the above buffer was suspended in 5 mM HEPES (pH 7.4), and the solution was dialyzed against 5 mM HEPES (pH 7.4). The dialyzed material was centrifuged at 100000*g* for 30 min, and the supernatant, which contained the purified SP-A, was stored in small aliquots at −20 °C. SDS−polyacrylamide gel electrophoresis (12% gel) was performed on samples of the SP-A solution according to the method of Laemmli (1970) followed by staining with Coomassie Blue. Under reducing conditions (5% β-mercaptoethanol in the sample buffer), a major band at approximately 36 kDa and a minor band at about 28 kDa were observed.

Monolayer Technique. Surface pressure (π)−area measurements were performed on a Langmuir trough, Applied Imaging (Dukesway Team Valley, Gateshead, Tyne and Wear, England), which employed a continuous Teflon ribbon barrier. Preliminary studies established that film leakage at the high surface pressures was not significant at least during the time scale of the current measurements; surface pressure in monolayers of DPPC which had been compressed just below or to the point of collapse at $\pi \approx 72$ mN/m did not change during an interval of about 20 min. Therefore, measures to prevent leakage, e.g., "priming" of the ribbon−water interfacial area by compressing monolayers of saturated phospholipids, were not taken. Surface tension was measured by the Wilhelmy plate method using a platinum plate roughened by scouring with emery paper. To spread SP-A, phospholipid, and SP-A/phospholipid monolayers, a solution containing 1-propanol and 0.5 M sodium acetate in equal volumes was employed. This solvent has been used for spreading of monolayers of DPPC or DMPC with other water-soluble proteins (Mita, 1989a). Protein or phospholipid was dissolved in the solvent at concentrations of approximately 0.2 mg/mL. In separate experiments, protein monolayers were formed by spreading of SP-A from 5 mM HEPES containing 0.1% amyl alcohol (Guastala, 1939). Films of either SP-A, or phospholipid, or their mixtures were formed by application of small drops of the spreading solution to the aqueous surface using a microsyringe (Hamilton Co., Reno, NV). A period of 15 min was allowed for evaporation of solvent and equilibration of the monolayers before the start of compression. The compression isotherms were obtained by continuous reduction of the film area at a rate of 40 cm^2/min. The total compression between the maximal area of 500 cm^2 to a minimal area of 100 cm^2 took 10 min.

Dynamic cyclic behavior of SP-A/DPPC monolayers was studied under two initial spreading conditions: (i) "surface dilute" conditions, where the amounts of initially-spread lipid−protein mixtures were small and the initial surface pressures before the start of compression were lower than 1 mN/m; and (ii) "surface excess" conditions, where the amounts of surface-active material initially-spread were in excess to those required for surface coverage by a monolayer (Notter et al., 1980a,b). In all experiments, the film area was continuously reduced and expanded 4 times. The relative change in the film area and the time for the compression−expansion cycles are specified further in the text.

The liquid subphase was 150 mM NaCl with or without 2 mM $CaCl_2$ in deionized doubly-distilled water. This concentration of Ca^{2+} is in the range reported for the extracellular aqueous lining layer of lung alveoli (Nielson, 1983). The pH of the subphase was adjusted to 7 immediately before each experiment, and it did not change by more than 1 pH unit during surface pressure−area measurements. The temperature was 22 ± 2 °C for all measurements. Whereas the surfactant in mammals functions at 37 °C, that found in air-breathing poikilotherms operates over a range of temperatures including 22 °C. In this temperature interval, both DPPC and DPPG are below their gel to liquid-crystalline transition temperature (T_c = 41 °C). While there may be some quantitative differences between measures of their interaction with SP-A at 22 and 37 °C, the major qualitative factors influencing the properties of the lipid−protein monolayers are likely to be the same at the two temperatures. Evaluation of lipid−protein interactions in mixed monolayers from DMPC with some water-soluble proteins (Mita, 1989a) supports the above concept.

The concentrations of the protein in the lipid−protein monolayers applied to the surfaces were expressed as weight percent of SP-A or "residue" fraction of protein amino acid residues, $X_r = N_r/(N_r + N_l)$, where N_r is the number of protein amino acid residues and N_l is the number of lipid molecules initially-spread in each monolayer. An assumption of a molecular weight of 35 000 and 248 residues per monomer of SP-A was used for all calculations (Weaver, 1988; Hawgood & Shiffer, 1991). The mean areas in the lipid−protein monolayers, A_{mean}, were calculated on the basis of molecules of lipid and amino acid residues of protein initially spread in the mixed films.

Analytical Methods. SP-A protein content was determined by the fluorescamine assay (Udenfriend et al., 1972) using bovine serum albumin as a standard. Concentrations estimated from the absorbance of the protein solutions at 277 nm and using the weight extinction coefficient for canine SP-A determined by King and MacBeth (1979) were within the range of concentrations determined by the fluorescamine assay. Aggregation of SP-A was assessed by monitoring the absorbance spectra of protein solutions in a Beckman DU-50 spectrophotometer at a wavelength of 300 nm using 1 cm path length cuvette (Haagsman et al., 1990). Measurements of circular dichroism of SP-A were made in a JASCO Model J-500A spectropolarimeter using 0.05 cm path length cells. SP-A concentration in either 5 mM HEPES or 1-propanol/0.5 M sodium acetate (1:1, v/v) was 0.2 mg/mL. The data were obtained by accumulating 32 scans from 260 to 200 nm, each run at a scan speed of 50 nm/min. Phospholipid concentrations were determined by measuring

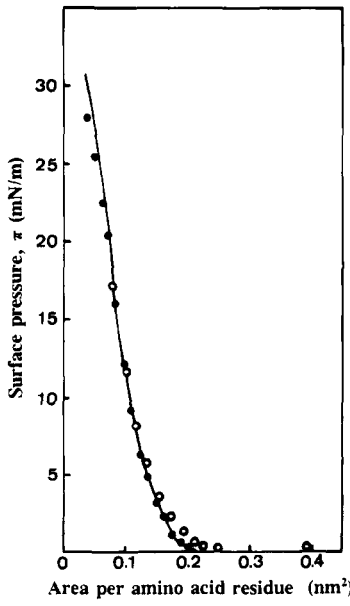

FIGURE 1: Isotherms of surface pressure versus area per amino acid residue for monolayers of SP-A spread on 0.15 M NaCl from 5 mM HEPES plus 0.1% amyl alcohol (continuous line) and 1-propanol/0.5 M sodium acetate (1:1, v/v) (empty and filled circles).

phospholipid phosphorus (Bartlett, 1959; Keough & Kariel, 1987).

RESULTS AND DISCUSSION

Spread Monolayers of SP-A. Temperature, pH, and buffer ionic strength influence the aggregation state of SP-A in solutions (Haagsman et al., 1990). In buffers of low ionic strength at physiological pH, SP-A is soluble in what is presumed to be its native octadecameric form (King et al., 1989). Calcium ions and high salt concentrations induced self-aggregation of SP-A (Haagsman et al., 1990). SP-A exhibited low intrinsic surface activity in forming adsorbed monolayers at the air−water interface (Hawgood et al., 1985a). In this work, we used different solvents to spread the monolayers of SP-A. The absorbance spectra recorded between 400 and 240 nm were identical for solutions of SP-A (0.1 mg/mL) in 5 mM HEPES and 1-propanol/0.5 M sodium acetate (1:1, v/v) (data not shown). This suggested that the use of 1-propanol/0.5 M sodium acetate did not cause aggregation of the protein. CD spectra of SP-A in 5 mM HEPES, comparable with published spectra for porcine (Casals et al., 1993) and canine SP-A (King et al., 1989), showed a shoulder at 220 nm and a maximum negative ellipticity at about 205 nm (data not shown). In 1-propanol/sodium acetate, the CD spectra of SP-A showed higher negative ellipticities between 235 and 215 nm, and this suggested an increased proportion of α-helical structure (data not shown). Due to solvent cutoff below 215 nm, it was not possible to assess effects of propanol/sodium acetate on the maximum negative ellipticity at 205 nm, which characterizes the structure of SP-A in neutral buffers at low ionic strength.

Protein monolayers were formed by application of SP-A in the buffer solution of low ionic strength in which SP-A was stored, 5 mM HEPES plus 0.1% amyl alcohol (Guastalla, 1939), to the surface of the subphase. Results from the surface pressure−area measurements are shown in Figure 1

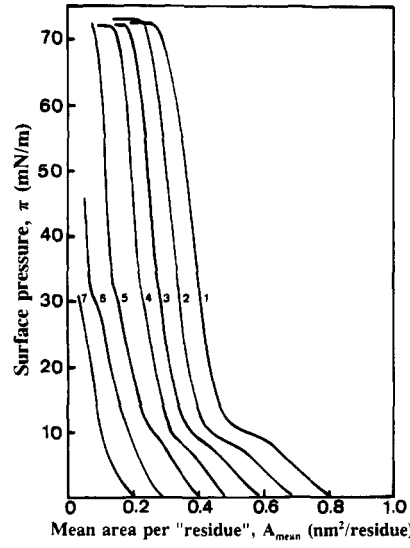

FIGURE 2: Surface pressure−area curves of spread monolayers of SP-A, DPPC, and their mixtures on 0.15 M NaCl. The initial concentration of SP-A in the monolayers, X_r, was 0.0 (1), 0.22 (2), 0.37 (3), 0.51 (4), 0.69 (5), 0.84 (6), and 1.0 (7).

(continuous curve). Similar isotherms for SP-A were obtained when the films were spread from a solution of 1-propanol/0.5 M sodium acetate (1:1, v/v) (Figure 1, filled circles). A 2-fold decrease in the amount of protein initially-spread from 1-propanol/0.5 M sodium acetate did not significantly alter the pressure−area curve for SP-A (Figure 1, empty circles). Experiments on the intrinsic surface activity of this spreading solvent showed that it did not change the surface tension of the aqueous subphase even when the surface was compressed to 20% of the initial area. The reproducibility of the $\pi(A)$ isotherms for the films of SP-A, spread from various aqueous solvents and on different initial surface areas, suggested that SP-A formed insoluble films at the air−water interface, and there was no property consistent with loss of protein into the subphase up to the point of film collapse. The limiting area, obtained by extrapolation of the linear part of the isotherm for SP-A to $\pi = 0$ mN/m, was approximately 0.14 nm² per amino acid residue, whereas the area at liftoff pressure corresponded to 0.20−0.22 nm²/amino acid residue. These values are consistent with those previously reported for spread films of other water-soluble proteins, such as bovine serum albumin (Taneva et al., 1981; Mita, 1989b) and human apolipoproteins A-I and A-II (Krebs et al., 1988).

Spread Monolayers of SP-A with DPPC. Surface pressure−area measurements in the absence of calcium in the subphase were performed on mixed films of SP-A plus DPPC spread from 1-propanol/0.5 M sodium acetate (1:1, v/v) (Figure 2). The $\pi(A)$ isotherms for films of DPPC spread from the propanol/acetate solution (Figure 2, curve 1) were shifted to lower areas compared to those obtained for chloroform-spread monolayers of the phospholipid (Taneva & Keough, 1994a). For example, at 40 mN/m we found an area of 0.39 ± 0.02 nm²/molecule for DPPC spread from the propanol/sodium acetate solution compared to 0.46 ± 0.01 nm²/molecule for chloroform-spread films. The molecular areas for DPPC spread from propanol/0.5 M sodium acetate (1:1, v/v) showed a high reproducibility of about ±5% usually reported for phospholipid films (Möhwald, 1990). A 2-fold decrease in the initial surface load, changes in the composition of the subphase (doubly-distilled water

instead of 0.15 NaCl), and an increased period of solvent evaporation and equilibration of the monolayer before the start of compression (30 min instead of 15 min) did not significantly affect the pressure−area curves for DPPC (data not shown). The reduction of about 15% in the molecular areas in DPPC monolayers spread from aqueous propanol/acetate compared to the chloroform-spread films likely resulted from different degrees of dispersion of DPPC in the spreading solvents. Concentrations of DPPC in the spreading solution of 1-propanol/0.5 M sodium acetate were about 10^{-4} M. We do not know the critical micelle concentration of DPPC in 1-propanol/0.5 M sodium acetate. However, concentrations of about 10^{-4} M were quite possibly above the cmc of DPPC in this solution, considering that the cmc of DPPC in 50% methanol/water solution is about 10^{-8} M (Smith & Tanford, 1972). The presence of aggregates in the aqueous propanol/sodium acetate solutions of DPPC may lead to partial dissipation of lipid into the subphase or incomplete dissociation of the DPPC aggregates, or both, during spreading of DPPC. Spreading was reproducible and substantial so that molecular areas close to those seen for chloroform-spread films were obtained.

Curves 2−6 in Figure 2 show pressure−area measurements on mixed films of SP-A plus DPPC on 0.15 M NaCl in the absence of calcium in the subphase. Each of the curves for the lipid−protein films which contained $\geq$5 wt % SP-A, or $X_r \geq 0.22$, displayed an inflection at $\pi \approx 30$ mN/m which was essentially independent of the initial monolayer composition. The inflections in the curves were readily seen as minima at about 30 mN/m in the plots of calculated surface elasticity [$E = -(d\pi/d \ln A)_T$] versus surface pressure (data not shown). A surface pressure of about 30 mN/m corresponded to the maximum surface pressure measured in the films of SP-A alone (Figure 1 and curve 7 of Figure 2). It might be argued that partial segregation of the components in the films occurred and squeeze-out of SP-A possibly commenced near $\pi \approx 30$ mN/m. The monolayers, which were consequently enriched in DPPC, attained maximum surface pressures of about 72 mN/m at the end of compression (for films of initial protein concentrations $X_r < 0.84$, or <50 wt % protein). Under similar experimental conditions, the squeeze-out pressures of the hydrophobic surfactant proteins, SP-B and SP-C, in the spread films containing DPPC were determined to be 45 and 50 mN/m, respectively (Taneva & Keough, 1994a,b). For films containing the hydrophobic proteins plus DPPC, the pressures for squeeze-out of proteins were higher than the collapse pressures for the proteins alone. Since the exclusion of SP-A in the films of SP-A plus DPPC occurred at pressures close to the collapse pressure of films of SP-A alone, it might be concluded that SP-A associated to a lesser extent with DPPC in spread monolayers than did the hydrophobic proteins.

To characterize the interaction between SP-A and DPPC in the binary films, the mean area per "residue" at constant surface pressure was plotted as a function of the initial protein concentration, X_r (Figure 3A). The positive deviations in the $A_{mean}(X_r)$ plots from ideal mixing, represented by the dashed lines in Figure 3, were consistent with partial miscibility of SP-A and DPPC in the monolayers. Similar behavior was seen in spread films of DPPC with the partially hydrophobic water-soluble hemoglobin and β-casein (Mita, 1989a), and for films of DPPC with the hydrophobic surfactant proteins SP-B and SP-C (Taneva & Keough,

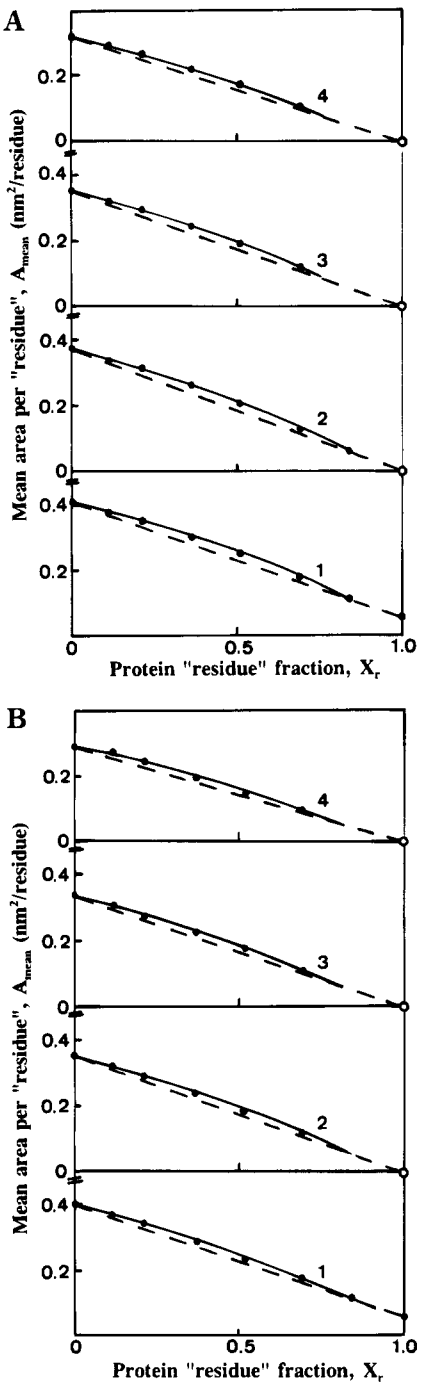

FIGURE 3: Mean area per "residue" at constant surface pressure versus initial protein concentration for SP-A/DPPC monolayers on 0.15 M NaCl (A) and 0.15 M NaCl plus 2 mM $CaCl_2$ (B). Surface pressure, π, mN/m: 25 (1), 45 (2), 55 (3), and 65 (4). The empty circles represent the values of A_{mean} extrapolated at the given surface pressure.

1994a,b). The nonideal behavior of the lipid−protein films could be interpreted in terms of perturbation of the hydrocarbon chain packing of phospholipid by the protein (Mita, 1989a), or vice versa. The former explanation seems more probable based on circular dichroism measurements which showed that in aqueous dispersions the spectra of canine SP-A in the presence of lipid were no different from the spectra of SP-A alone (Haagsman et al., 1989).

Studies of interactions of SP-A with DPPC in aqueous dispersions (King & MacBeth, 1981; King et al., 1983) or with DPPC immobilized on thin-layer chromatography plates

(Kuroki & Akino, 1991) have suggested that the principal mode of interaction between SP-A and DPPC is via hydrophobic forces. Ross et al. (1986) have proposed the importance of a putative amphipatic α-helix region of SP-A for protein−lipid interactions.

The expansion in the SP-A/DPPC films seen at surface pressures above the exclusion pressure for SP-A (Figure 3A, 2−4) could be attributed to the presence of some residual protein in the films after squeeze-out of part of the initially-spread SP-A. Therefore, the collapse phase(s) formed at 72 mN/m possibly contained some SP-A which may be expected to influence respreading properties of the lipid−protein monolayers. Our previous surface pressure−area measurements on spread films of surfactant proteins SP-B or SP-C with DPPC indicated that small amounts of the hydrophobic proteins were present in the phospholipid films compressed to $\pi \approx 72$ mN/m (Taneva & Keough, 1994a,b,d). Thus, the surface pressure−area measurements performed *in vitro* suggest that the three pulmonary surfactant-associated proteins could be present at low levels in the alveolar surface film in the whole range of surface pressures between 40 and 70 mN/m measured for the alveolar interface during the breathing cycle (Schürch, 1982). The implication of these observations for the complex process of formation, refinement, and renewal of the surface film in the alveoli remains unknown. The presence of the hydrophobic SP-B and SP-C in the collapse phase(s) of DPPC improved the respreading properties of DPPC (Taneva & Keough, 1994d). Efficient respreading of DPPC from two- or three-dimensional phases has been considered important for the replenishment of phospholipid in the alveolar monolayer during breathing and for exogenous treatment of pulmonary surfactant insufficiency *in vivo* (Turcotte et al., 1977).

The effect of calcium ions on the properties of the SP-A/DPPC films was studied. Calcium binds to SP-A (Haagsman et al., 1990). Measurements of the intrinsic fluorescence of intact canine SP-A showed that interaction with calcium resulted in small or no changes in the conformation of the protein (King et al., 1983; Haagsman et al., 1990). Calcium binding induced conformational changes in a proteolytic fragment of SP-A which binds carbohydrates in a Ca^{2+}-dependent fashion (Haagsman et al., 1990). The $\pi(A)$ isotherms for the films of SP-A measured in the presence of either 2 mM $CaCl_2$ or 2 mM EDTA in the saline subphase (data not shown) were similar to each other and to the isotherms shown in Figure 1. From the surface pressure measurements in this study, it appeared that calcium in the subphase did not have a significant effect on the conformation of the intact porcine SP-A in the spread films, at least to the point of affecting the monolayer characteristics of the protein.

Ca^{2+} in the subphase did not affect the isotherms for the films of DPPC spread from propanol/0.5 M sodium acetate (data not shown). This observation was consistent with the low capability of the divalent ions to bind to the lecithin head group (Hauser & Phillips, 1975) and to affect molecular packing in monolayers of phosphatidylcholines (Shah & Schulman, 1965). The plots of the mean areas in the SP-A/DPPC films versus initial protein concentration were essentially Ca^{2+}-independent, as can be seen in the comparison of Figure 3A and Figure 3B, and this implied that calcium in the subphase did not modify the interactions between SP-A and DPPC in the spread monolayers.

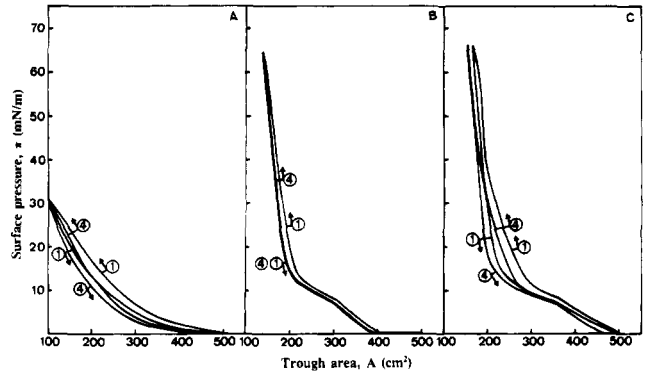

FIGURE 4: Cyclic compression−expansion isotherms for monolayers of SP-A (A), DPPC (B), and a mixture of the two containing 17 wt % SP-A, or $X_r = 0.52$ (C). The subphase was 0.15 M NaCl. Cycles 1 and 4 are shown. The rate of compression and expansion was 200 cm²/min.

Effect of SP-A on the Properties of DPPC Monolayers during Cyclic Compression and Expansion. The cyclic compression−expansion isotherms for SP-A, shown in Figure 4A, indicated that the maximum surface pressure of about 30 mN/m attained at the end of compression did not change significantly when the films were repeatedly compressed and expanded. This suggested that little or no permanent loss of protein due to desorption occurred under these experimental conditions. During compression of some protein monolayers, reversible displacement and reentry of segments of the molecules have been suggested to take place (MacRitchie, 1981). These processes, having slow kinetics with relaxation times of the order of 60 min at $\pi = 25$ mN/m (MacRitchie, 1981), i.e., longer than the cycling frequency used in the present study, possibly contributed to the observed hysteresis in the cyclic $\pi(A)$ curves for SP-A. Cyclic pressure−area isotherms for spread films of SP-B or SP-C also showed reversible behavior under similar experimental conditions (Taneva & Keough, 1994d).

Figure 4B,C shows the pressure−area curves for films of DPPC alone and films of DPPC plus 17 wt % SP-A ($X_r = 0.52$) which were compressed and expanded 4 times between 0 and 65 mN/m. The maximum surface pressure at the end of compression was higher than the collapse pressure of SP-A, defined as a maximum pressure attained in the protein films (~30 mN/m) under similar experimental conditions (Figure 4A), and it did not exceed the collapse pressure for the films of pure DPPC (72 mN/m). Films of DPPC (Figure 4B) showed a small hysteresis in the pressure−area loops, similar to that seen in films spread from chloroform (Taneva & Keough, 1994d). The addition of 17 wt % SP-A, $X_r = 0.52$, was accompanied by an increase in the hysteresis area and this may be accounted for by exclusion and reentry of the protein moiety during cyclic compression−expansion of the mixed films. The fourth compression isotherm for the SP-A/DPPC films in Figure 4C was shifted to low areas, consistent with some loss of material during repetitive compression of the films. The plot of surface elasticity versus surface pressure calculated for the fourth compression isotherm in Figure 4C showed a minimum at $\pi \approx 30$ mN/m, associated with a squeeze-out of SP-A, very similar to the $E(\pi)$ plot for the first compression isotherm (data not shown). This observation suggested that SP-A was still present in the monolayers after the films had been repeatedly compressed and expanded. Similar measurements on spread

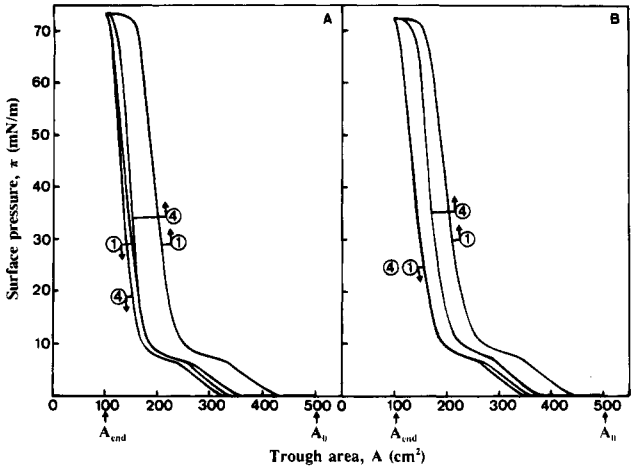

FIGURE 5: Cyclic compression–expansion isotherms for monolayers of DPPC (A) and DPPC plus 2.5 wt % SP-A, or $X_r = 0.12$ (B), formed at initial "surface dilute" concentrations. The subphase was 0.15 M NaCl. Cycles 1 and 4 are shown.

Table 1: Respreading Characteristics of the Monolayers of DPPC plus Surfactant Protein SP-A under Initial "Surface Dilute" Spreading Conditions[a]

monolayer	concentration of SP-A wt %	concentration of SP-A X_r[b]	initial spreading surface pressure,mN/m	collapse plateau length ratios[c] 2/1	collapse plateau length ratios[c] 4/1
DPPC		0.05	<1	0.61	0.41
SP-A/DPPC	1.0	0.12	<1	0.75	0.60
SP-A/DPPC	2.5	0.22	<1	0.82	0.68
SP-A/DPPC	5.0		<1	0.81	0.69

[a] Each monolayer contained an identical amount of DPPC, corresponding to 0.88 nm² per molecule of DPPC at the start of the first compression–expansion cycle. In each experiment, for each successive cycle, a maximum surface pressure of 71 ± 1 mN/m was attained at the end of compression, and surface pressures of about 0 mN/m were reached at the ends of expansions. [b] X_r is the mole ("residue") fraction of amino acid residues of SP-A in the mixed films. [c] Ratios of collapse plateaus for cycle 2/cycle 1 and cycle 4/cycle 1.

films of DPPC with SP-B or SP-C were consistent with reversible exclusion and reentry of the hydrophobic proteins (Taneva & Keough, 1994d).

Dynamic cyclic surface pressure–area measurements on DPPC and SP-A/DPPC films, compressed beyond the collapse point of DPPC, were performed under two initial surface conditions. In a first series of experiments, each of the films of DPPC alone and those of DPPC plus SP-A contained an equal amount of DPPC corresponding to 0.88 nm² per molecule of DPPC at the start of compression. This surface load gave rise to initial surface pressures lower than 1 mN/m. Cycling time was 240 s, and relative change in the monolayer area was 80%. The results in Figure 5A showed that DPPC had a low ability to respread from collapse phase(s) and this led to a large hysteresis in the first compression–expansion cycle and considerable displacement of the fourth compressional isotherm to low areas. The collapse plateau length ratio criterion, introduced by Notter et al. (1980a,b), was used as a measure for the extent of respreading of the collapse phase(s). The collapse plateau length ratios for DPPC, shown in Table 1, were comparable with those determined for DPPC films spread from chloroform (Taneva & Keough, 1994d). The values in Table 1 indicate a low tendency of DPPC to respread from collapse phase(s). Irreversible loss of molecules into the subphase or formation of surface collapse phases with very slow respreading kinetics may account for the poor respreadability of DPPC compressed beyond collapse (Turcotte et al., 1977; Snik et al., 1978).

Figure 5B shows the results for the cyclic compression–expansion of films of DPPC plus 2.5 wt % SP-A, or $X_r = 0.12$. Similar measurements were performed on SP-A/DPPC films which contained 1 or 5 wt % SP-A, $X_r = 0.05$ and $X_r = 0.22$, respectively. As discussed earlier, the $A_{mean}(X_r)$ diagrams for the SP-A/DPPC films in Figure 3A suggested that, independent of the initial composition, part of SP-A was present in the films at surface pressures above the exclusion pressure of SP-A and therefore some of the protein was possibly incorporated into the collapse phase(s) formed at 72 mN/m.

The lengths of the collapse plateaus for the SP-A/DPPC films were determined, and their ratios were recorded in Table 1. DPPC films which contained SP-A showed superior respreading over those of DPPC alone. The effect of SP-A on the respreadability of DPPC reached a point of saturation at about 2.5 wt % SP-A, or $X_r = 0.12$. Higher amounts of SP-A did not further improve respreading. A concentration of 5 wt % SP-A in the phospholipid film is relevant to the amount of protein reported for surface-active material from lung lavage fluids (Sueishi & Benson, 1981).

Values of the ratios for cycle 2/cycle 1 and cycle 4/cycle 1 for the DPPC films containing 2.5 wt % SP-A in comparison to those for DPPC containing an equal weight of either SP-B or SP-C (Taneva & Keough, 1994d) suggested that the ability of the surfactant-associated proteins to promote respreading of DPPC was in the order SP-B > SP-A > SP-C under these experimental conditions. At higher protein concentrations, e.g., 5 wt %, the proteins facilitated postcollapse respreading of the protein/DPPC monolayers in the order SP-B > SP-C > SP-A. It is worth noting that similar to SP-A, the presence of the hydrophobic proteins in the films of DPPC caused perturbation of the molecular packing in the phospholipid films and this effect seems to correlate with the ability of these additives to improve postcollapse respreading of DPPC (Taneva & Keough, 1994d).

In a second series of experiments, the films of DPPC, alone or mixed with SP-A, were formed under initial "surface excess" conditions. At the start of the first cycle, each of the films of DPPC alone or DPPC plus SP-A contained an equal amount of DPPC, corresponding to 0.24 nm² per molecule of DPPC. This surface load gave rise to initial surface pressures of about 46 mN/m. Films were continuously compressed and expanded 4 times between the initial area A_{excess} and the minimum area A_{end} (Figure 6A). The cycling time was 30 s, and the relative change in the film area was 57%. Figure 6A shows the isotherms for the cyclic compression–expansion of the films of pure DPPC. The "surface excess" films of DPPC showed poor respreadability as indicated by the absence of a collapse plateau region in the fourth cycle. The collapse plateau length ratios for DPPC, recorded in Table 2, showed inferior respreading of the films compared to their "surface dilute" counterparts (Table 1). Similar behavior was seen in the DPPC films formed from chloroform (Taneva & Keough, 1994d). Differences in structures of the phospholipid monolayers spread at the two initial surface pressures and different conditions of performing the experiments, e.g., surface pressure at the

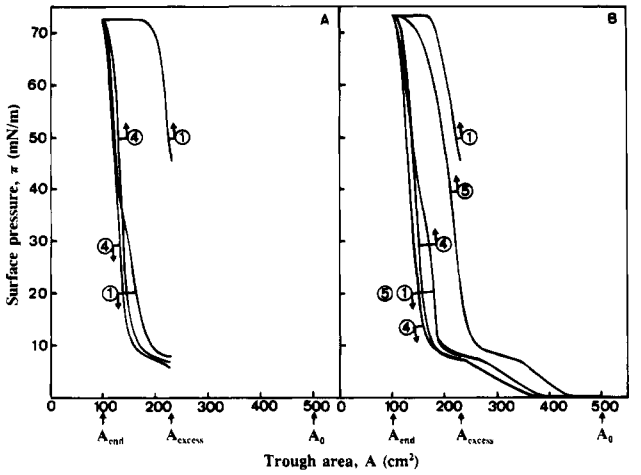

FIGURE 6: Cyclic compression−expansion behavior of "surface excess" films of DPPC (A) and DPPC plus 2.5 wt % SP-A, or X_r = 0.12 (B). The subphase was 0.15 M NaCl. Cycles 1 and 4 are shown. Figure 6B shows the fifth compression−expansion curve which was measured after expansion of the SP-A/DPPC films to zero surface pressure at the end of the fourth cycle.

Table 2: Respreading Characteristics of the Monolayers of DPPC plus Surfactant Protein SP-A under Initial "Surface Excess" Spreading Conditions[a]

monolayer	concentration of SP-A wt %	concentration of SP-A X_r[b]	initial spreading surface pressure,[c] mN/m	collapse plateau length ratios 2/1	collapse plateau length ratios 4/1
DPPC		0.12	45.6	0.44	0
SP-A/DPPC	2.5	0.22	45.8	0.43	0.0 (0.69)
SP-A/DPPC	5.0	0.72	46.3	0.43	0
SP-A/DPPC	10.0		45.8	0.42	0

[a] In each experiment, for each successive cycle, the surface pressure was 72 ± 1 mN/m at the end of compression, and it was higher than 0 mN/m at the end of expansion. The value in parentheses represents the ratio of the collapse plateau lengths of cycle 5/cycle 1 determined after the expansion of the film to π = 0 mN/m at the end of the fourth cycle (see Figure 6B). [b] X_r is the mole ("residue") fraction of amino acid residues of SP-A in the mixed films. [c] The initial spreading pressure corresponded to 0.24 nm² per molecule of DPPC.

start of each consequent cycle, possibly accounted for this observation (Taneva & Keough, 1994d).

The dynamic cyclic behavior of "surface excess" films of DPPC containing 2.5 wt % SP-A (X_r = 0.12), shown in Figure 6B, indicated that the addition of the protein had no significant effect on the respreadability of DPPC (cycles 1 and 4). A 4-fold increase in the concentration of SP-A did not improve the respreading of the films (Table 2). Contrary to these observations, the hydrophobic surfactant proteins SP-B and SP-C did enhance the respreading of the protein/DPPC films formed under similar initial spreading conditions (Taneva & Keough, 1994d). The absence of an effect of SP-A on the respreading of DPPC implied that SP-A possibly was not incorporated into the monolayer, and hence into the collapse phase of DPPC. Perhaps, SP-A was selectively squeezed out from the SP-A/DPPC monolayers, i.e., the protein formed a separate collapse phase, during the spreading of the "surface excess" monolayers at $\pi \approx 46$ mN/m, which was significantly higher than the collapse pressure of SP-A alone (≈30 mN/m) or the exclusion pressure of SP-A in the SP-A/DPPC monolayers (≈30 mN/m). In such a case, at the start of the first cycle of compression−expansion of the films, we probably dealt essentially with a monolayer of "pure" DPPC which showed respreading characteristics similar to those for the "excess" films of DPPC alone.

In a previous study, we showed that expansion of the "surface excess" films that had been compressed beyond collapse to low, near-zero, surface pressures increased the respreading of postcollapse films of DPPC plus either SP-B or SP-C formed at initial "excess" concentrations (Taneva & Keough, 1994d). To check whether this procedure would enhance the respreading of "surface excess" films of SP-A/DPPC, the fourth expansion of SP-A/DPPC films in Figure 6B was not halted at the area A_{excess}, but continued to the larger area A_0, so that a zero surface pressure was attained at the end of expansion. Then the films were compressed to a minimum area A_{end} and reexpanded to the area A_0 (cycle 5 in Figure 6B). The displacement of the fifth compression curve to higher areas, compared to the fourth one, implied that the expansion of the film to low (zero) pressures at the end of the fourth cycle enhanced the respreading of the collapse phase. The ratio cycle 5/cycle 1 for the SP-A/DPPC films in Figure 6B, shown in parentheses in Table 2, was comparable to that for the films of similar initial composition formed under "surface dilute" conditions (Table 1). Before, we suggested that expansion of the "surface excess" films to near-zero surface pressures possibly led to dissociation of molecular aggregates formed during spreading and collapse of the films, and to reorientation of the molecules at the low pressures (Taneva & Keough, 1994d). In addition to these processes, an enhanced miscibility between SP-A and DPPC and incorporation of the protein into the lipid monolayer at the low surface pressures, and therefore into the lipid collapse phase(s) formed at the end of compression, may also account for the improved respreading of the "surface excess" films that had been expanded to zero surface pressure.

Spread Monolayers of SP-A with DPPG. SP-A is characterized by a charge heterogeneity, with isoforms ranging from a p*I* of 4.8 to a p*I* of 5.3 (Sueishi & Benson, 1981; Hawgood et al., 1985). The experiments in this study were performed at a pH of 6−7, and one could expect that the spread films of SP-A carried a net negative charge. DPPG forms fully ionized monolayers at pH >4 for subphase ion concentrations of NaCl above 10 mM (Lakhdar-Ghazal et al., 1983). Therefore, at pH 7 direct electrostatic forces between the protein and lipid bearing a similar charge could possibly affect the interfacial properties of the mixed films. Though the PG of surfactant contains both saturated and unsaturated species (Yu et al., 1983), DPPG was chosen in the present study for examination of electrostatic effects between SP-A and PG, since in its sodium salt DPPG displays monolayer and bilayer characteristics similar to those of DPPC, and confounding factors such as unsaturation would not complicate interpretation of results.

Surface pressure−area curves for SP-A, DPPG, and mixtures of the two in the absence of calcium in the subphase are shown in Figure 7. A reduction in the molecular areas of about 12% was seen in the isotherm of DPPG in Figure 7 compared to that measured for monolayers spread from chloroform/methanol on subphases of similar ionic strength and pH (Sacrè & Tocanne, 1977; Taneva & Keough, 1994a). SP-A, at low concentrations, $X_r \leq 0.52$, or ≤17 wt %, did not interfere with the ability of DPPG films to attain a maximal surface pressure of about 72 mN/m (Figure 7, curves 2−5). The plots of calculated surface elasticities

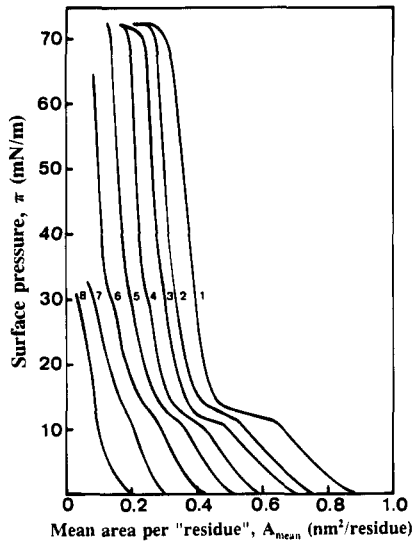

FIGURE 7: Surface pressure–area isotherms for spread monolayers of SP-A with DPPG on 0.15 M NaCl. Initial concentration of SP-A, X_r: 0.0 (1), 0.11 (2); 0.22 (3), 0.37 (4), 0.52 (5), 0.69 (6), 0.84 (7), and 1.0 (8).

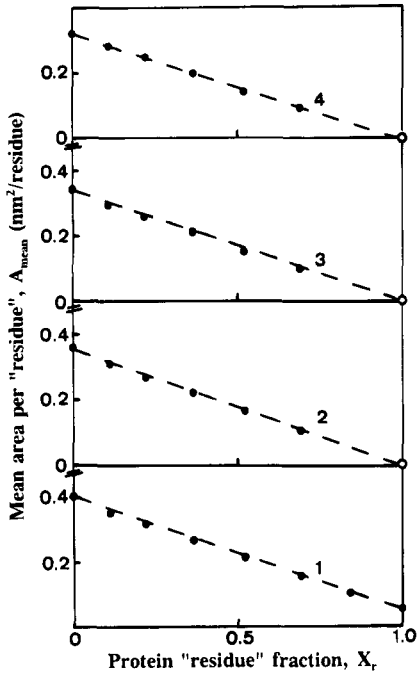

FIGURE 8: Mean area per "residue" in the SP-A/DPPG films on 0.15 M NaCl versus initial protein concentration at constant surface pressure, π, mN/m: 25 (1), 45 (2), 55 (3), and 65 (4). The empty circles represent the values of A_{mean} extrapolated at the given surface pressure.

versus surface pressure for the SP-A/DPPG films which contained ≥5 wt % SP-A, or $X_r \geq 0.22$, displayed minima at $\pi \approx 30$ mN/m, likely associated with squeeze-out of SP-A (data not shown).

The additive behavior of the mean areas per "residue" in the SP-A/DPPG films at constant surface pressure in Figure 8 was consistent with either ideal mixing or complete demixing of the protein and lipid. The observation of two collapse pressures in the SP-A/DPPG films—a discontinuity at $\pi \approx 30$ mN/m corresponding to protein collapse, and a second collapse plateau at $\pi \approx 70$ mN/m, associated with DPPG collapse—suggested that immiscibility of the components was likely responsible for the additive behavior of

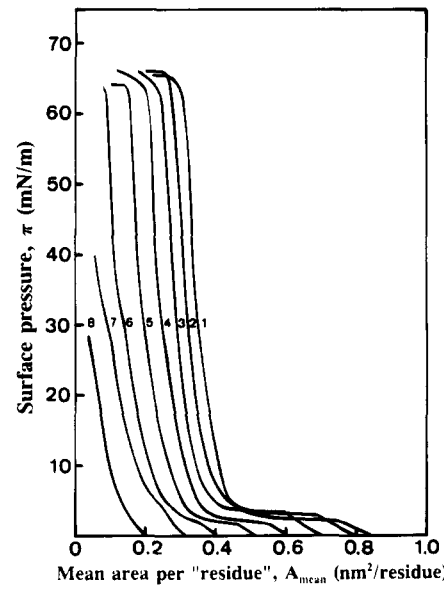

FIGURE 9: $\pi(A_{mean})$ isotherms of SP-A/DPPG monolayers on 0.15 M NaCl plus 2 mM $CaCl_2$ in the subphase. Initial concentration of SP-A, X_r: 0.0 (1), 0.12 (2), 0.22 (3), 0.37 (4), 0.52 (5), 0.69 (6), 0.84 (7), and 1.0 (8).

the mean areas. Electrostatic repulsion between SP-A and DPPG possibly prevented them from favorable orientation in the monolayers so that nonpolar interaction did not occur. This property of the films of DPPG plus SP-A was contrary to the effects seen in the films of DPPC plus SP-A (Figure 3) although the two phospholipids had hydrocarbon chains of identical length and degree of saturation.

Measurements of the intrinsic fluorescence of SP-A in the presence of phospholipid vesicles showed the absence of interactions between SP-A and DPPG, as opposed to DPPC (Casals et al., 1993). Likewise, SP-A exhibited almost no binding to DPPG compared to DPPC in experiments where the phospholipids were immobilized on silica gel plates (Kuroki & Akino, 1991). These results, and the data in the present study, reveal the importance of the polar head groups of phospholipids for directing their interactions with SP-A.

Surface pressure–area measurements were performed on SP-A/DPPG films in the presence of 2 mM $CaCl_2$ in the subphase (Figure 9). Calcium ions acted to decrease both the pressure required for the transition from the liquid-expanded to the liquid-condensed state of DPPG and the collapse pressure of the films (compare curve 1 in Figure 9 and curve 1 in Figure 7). These effects seen in the presence of Ca^{2+} were consistent with the ability of the divalent ions to neutralize the phosphatidylglycerol head group charges and hence to increase the lateral packing density of the phospholipid monolayer (Verkleij et al., 1974; Hauser et al., 1975). Calcium ions exerted similar condensing effects on the films of DPPG plus SP-A (Figure 9, curves 2–7, compared to Figure 7, curves 2–7) and had no effect on the monolayer of SP-A alone (compare Figure 9, curve 8, and Figure 7, curve 8). Though SP-A binds Ca^{2+} (Haagsman et al., 1990), comparison of the association constants of Ca^{2+} with SP-A (Haagsman et al., 1990) and with eggPG (Lau et al., 1981) suggests a weaker interaction of Ca^{2+} with SP-A than with DPPG.

Plots of the mean area per "residue" versus initial protein concentration for the SP-A/DPPG films in the presence of calcium were constructed (Figure 10) and compared with

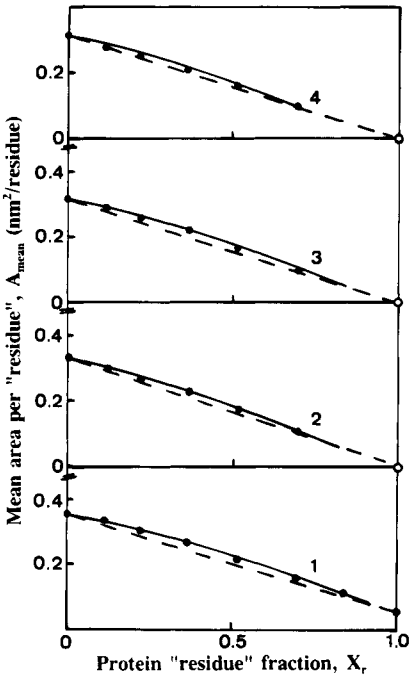

FIGURE 10: Mean area per "residue" in the SP-A/DPPG monolayers versus initial composition in the presence of 0.15 M NaCl and 2 mM $CaCl_2$ in the subphase. Surface pressure, π, mN/m: 25 (1), 45 (2), 55 (3), and 65 (4). The empty circles represent the values of A_{mean} extrapolated at the given surface pressure.

those obtained in the absence of Ca^{2+} (Figure 8). The nonideal behavior of the $A_{mean}(X_r)$ plots in Figure 10 suggested that neutralization of the charges of DPPG (and SP-A) by calcium led to improved miscibility between SP-A and DPPG. The $A_{mean}(X_r)$ plots for the mixtures of SP-A with DPPG in the presence of calcium (Figure 10) were very similar to those for SP-A with DPPC (Figure 3) where van der Waals interactions between the hydrophobic parts of the molecules possibly determined the behavior of the films. Ternary interactions between SP-A, Ca^{2+}, and phosphatidylglycerols have been suggested as a possible mechanism of association between SP-A and the acidic phospholipid in phospholipid dispersions (King & MacBeth, 1981).

Spread Monolayers of SP-A with a DPPC/DPPG Mixture. Spread monolayers of SP-A with a mixture of DPPC and DPPG in the absence of Ca^{2+} in the subphase were studied (Figure 11). The molar ratio between the two phospholipids was 7:3 in all SP-A/(DPPC/DPPG) films. The ternary SP-A/(DPPC/DPPG) films displayed features similar to those of the binary SP-A/DPPC (Figure 2) and SP-A/DPPG (Figure 7) films. The plots of the mean areas per "residue" in the SP-A/(DPPC/DPPG) films versus initial protein concentration showed no deviations from the additivity law at all surface pressures studied (Figure 12). The mean areas in the binary films of SP-A with DPPC showed nonideal behavior, consistent with apolar Ca^{2+}-independent interactions between SP-A and the zwitterionic DPPC (Figure 3). On the other hand, the $A_{mean}(X_r)$ diagrams for the binary films of SP-A with DPPG in the absence of calcium showed additive behavior, consistent with immiscibility of the components (Figure 8). In the ternary SP-A/(DPPC/DPPG) films, the addition of 30 mol % of negatively charged DPPG removed the evidence for interactions between SP-A and DPPC as indicated by the additivity of the $A_{mean}(X_r)$ plots in Figure 12. Therefore, electrostatic effects between SP-A and

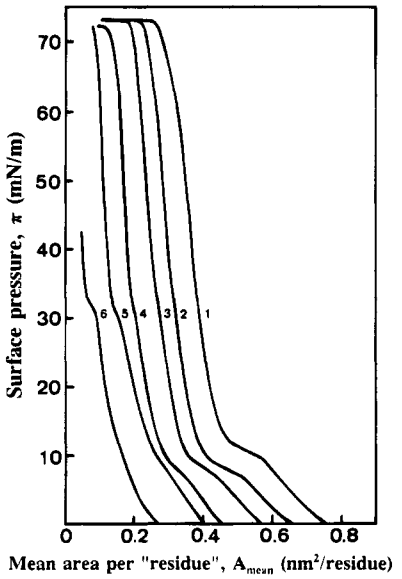

FIGURE 11: $\pi(A_{mean})$ isotherms for SP-A/(DPPC/DPPG) (7:3, mol/mol) monolayers on 0.15 M NaCl. Initial protein concentration, X_r: 0.0 (1), 0.22 (2), 0.37 (3), 0.52 (4), 0.69 (5), and 0.84 (6).

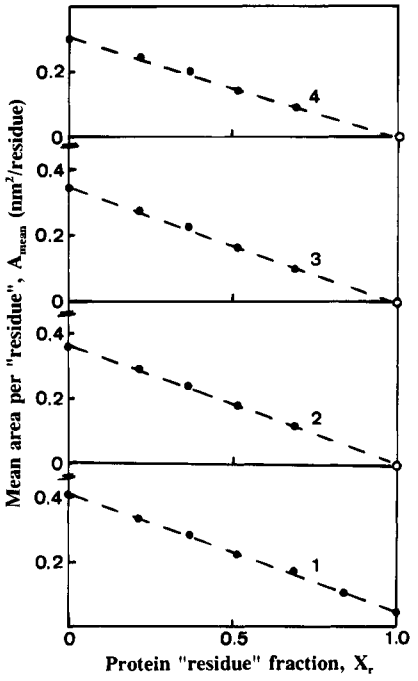

FIGURE 12: Mean area per "residue" in the SP-A/(DPPC/DPPG) (7:3, mol/mol) films on 0.15 M NaCl as a function of initial protein concentration. Surface pressure, π, mN/m: 25 (1), 45 (2), 55 (3), and 65 (4). The empty circles represent the values extrapolated at the given surface pressure.

DPPG seemed to govern the behavior of the ternary films.

The reduction observed in the miscibility of SP-A with the phospholipid mixture containing the negatively charged DPPG was in accord with results from intrinsic fluorescence measurements in the absence of Ca^{2+}, which indicated a weaker interaction of porcine SP-A with DPPC/DPPG vesicles compared to pure DPPC vesicles (Casals et al., 1993).

The effect of Ca^{2+} on the mixing properties of SP-A with DPPC/DPPG (7:3, mol/mol) was examined. Ca^{2+} exerted a condensing effect on the films of DPPC/DPPG and their mixtures with SP-A (compare Figure 13 and Figure 11).

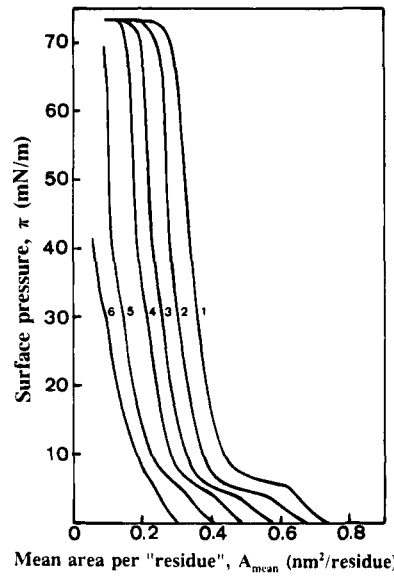

FIGURE 13: $\pi(A_{mean})$ curves for SP-A/(DPPC/DPPG) (7:3, mol/mol) monolayers on 0.15 M NaCl plus 2 mM $CaCl_2$ in the subphase. Initial concentration of SP-A, X_r: 0.0 (1), 0.22 (2), 0.37 (3), 0.52 (4), 0.69 (5), and 0.84 (6).

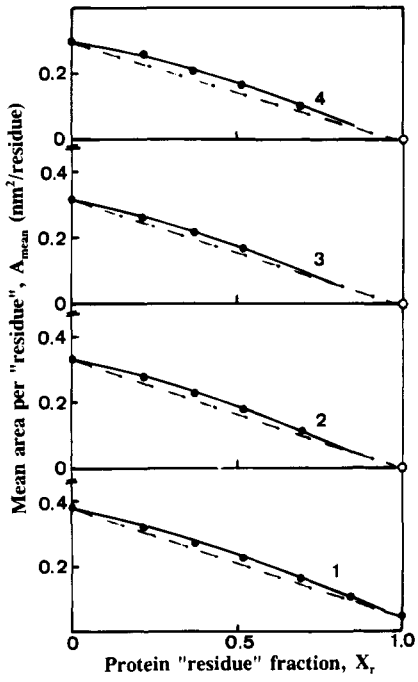

FIGURE 14: Mean area per "residue" in the SP-A/(DPPC/DPPG) (7:3, mol/mol) monolayers on 0.15 M NaCl plus 2 mM $CaCl_2$ versus initial protein concentration. Surface pressure, π, mN/m: 25 (1), 45 (2), 55 (3), and 65 (4). The empty circles represent the values of A_{mean} extrapolated at the given surface pressure.

During compression of the SP-A/(DPPC/DPPG) films which contained more than 5 wt % SP-A ($X_r \geq 0.22$), squeeze-out of SP-A at about 30 mN/m was suggested by the $E(\pi)$ plots (data not shown). The positive deviations from the additive law in the $A_{mean}(X_r)$ plots, shown in Figure 14, indicated that the addition of Ca^{2+} to the subphase restored interactions between the protein and DPPC/DPPG in the monolayers. Screening of the electrostatic repulsion between SP-A and DPPG by calcium likely improved the miscibility of SP-A with the phospholipid mixture. This observation of a Ca^{2+}-dependent interaction of SP-A with the mixture of DPPC/DPPG was consistent with findings that SP-A triggered aggregation of aqueous dispersions of DPPC/DPPG, or phospholipid mixtures containing phosphatidylglycerols, and promoted the formation of a surface film only in the presence of calcium ions (King et al., 1983; Hawgood et al., 1985; Schürch et al., 1992).

CONCLUSIONS

SP-A formed insoluble monolayers at the air−water interface when it was spread from aqueous solutions. SP-A interacted with the major phospholipid of pulmonary surfactant, DPPC, when they were spread in monolayers at the air−water interface. These interactions were independent of the presence of Ca^{2+} in the subphase and possibly were of nonpolar origin. The exclusion pressure for SP-A during compression of the films with DPPC was determined to be about 30 mN/m, close to the collapse pressure of SP-A alone. Due to lipid−protein interactions, part of the initially-spread SP-A appeared to remain in SP-A/DPPC films that were compressed to surface pressures of about 70 mN/m, and it possibly was incorporated into the collapse phase. SP-A enhanced the respreading of SP-A/DPPC films over that of films of DPPC alone only when the lipid−protein films were formed at initial low ("dilute") concentrations, and the protein had no effect on respreading of the films that were formed at initial high ("excess") concentrations. A possible explanation for this behavior of the "surface excess" films is to assume that SP-A was not incorporated into the initial monolayers spread at high surface pressures above the collapse pressure of SP-A alone and therefore the protein was not included into the DPPC collapse phase(s) formed at maximum compression of the films. SP-A, at a physiologically relevant concentration, e.g., 5 wt %, was less effective in promoting respreading of collapse phases of DPPC than were the hydrophobic surfactant proteins SP-B and SP-C (Taneva & Keough, 1994d).

The interactions of SP-A with DPPG, or with DPPC/DPPG (7:3, mol/mol) were Ca^{2+}-dependent. In the absence of Ca^{2+}, the mean monolayer areas in SP-A/DPPG and SP-A/(DPPC/DPPG) films showed behavior that was consistent with segregation of the components. Electrostatic repulsion between SP-A and DPPG or DPPC/DPPG possibly accounted for the immiscibility of the protein and lipid. The addition of calcium to the subphase, presumably through screening of electrostatic repulsion between the negatively charged DPPG and SP-A, induced interactions between the protein and phospholipids that likely occurred through hydrophobic forces. These results implied a significant role for Ca^{2+}-dependent electrostatic effects between SP-A and phosphatidylglycerols in governing the overall interactions of SP-A with mixtures that contain acidic phospholipids.

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