PULMONARY SURFACANT stabilizes the lung by reducing surface tension at the air-alveolar fluid interface of the alveoli. Surfactant forms interfacial films that reduce alveolar surface tension to very low values at end expiration, thereby countering alveolar collapse. Although the molecular structure and composition of the surfactant film in vivo are not known, recent electron-microscopic evidence suggests that the surfactant lining the alveolar fluid surface includes multilayer films (29, 31, 33). These multilayers are presumed to consist of a single monolayer (i.e., the outer leaflet), with underlying multilayers acting as a surface-associated surfactant “reservoir” (33). Disaturated phospholipids such as dipalmitoylphosphatidylcholine (DPPC) can pack into a gel phase in monolayer films under compression and reduce interfacial surface tension to near 0 mN/m at 37°C. In contrast, fluid unsaturated phospholipid components of pulmonary surfactant cannot form stable films capable of generating surface tensions near zero at physiological temperatures. Surface tensions approaching 0 mN/m have been measured in the lung in situ during deflation (30).

In addition to the major component, DPPC, surfactant contains significant amounts of unsaturated phosphatidylcholine (PC), phosphatidylglycerol (PG), other lipids, and small amounts of surfactant-associated protein (SP) A, SP-B, SP-C, and SP-D (see Refs. 2, 13 for reviews). SP-B is a 17-kDa hydrophobic dimeric protein that has cationic amphipathic helices at physiological pH (14). Genetic SP-B deficiency (21) and targeted disruption of the SP-B gene (5) lead to fatal respiratory distress. Such genetic SP-B deficiencies are associated with inappropriate SP-C processing, resulting in combined SP-B and SP-C deficiency and making it impossible to conclude which surfactant apoprotein, if either, is essential. However, another study (35) has shown that administration of antibodies directed against SP-B is essential. However, another study (35) has shown that administration of antibodies directed against SP-B produces respiratory failure in animals, suggesting a clear importance of this protein in surfactant and lung physiology. To alleviate such conditions, various lipid-SP-B and lipid-SP-B-like peptide mixtures have been examined in artificial surfactants (11, 13, 17).

Many studies have indicated that SP-B enhances the surface active properties of surfactant phospholipids and has the potential for interactions with negatively charged surfactant lipids such as PG. In vitro studies (2, 11, 13, 15) with SP-B in model surfactant lipid systems have suggested that SP-B interacts with the head groups of acidic surfactant lipids and enhances the adsorption, spreadability, and surface insertion of the surfactant lipids, including DPPC, by as yet unclear mechanisms. It appears that SP-B is more potent in some surface processes, such as in inserting phospholipids into preformed films above equilibrium surface tension, than SP-C (22). In some of the model system studies, solvent-spread monolayers of the lipid-SP-B mixtures at the air-water interface were maintained.
under nonphysiological conditions and high protein-to-lipid ratios were used compared with their real amounts in surfactant (18, 20, 36). Although these model studies have given valuable information on the structure, properties, and interactions of the protein with the lipid systems, relatively few studies of model lipid-hydrophobic protein systems under physiological conditions exist to date (24, 41). In light of the recent view that pulmonary surfactant in the alveoli may contain multilayers (or a monolayer film with underlying reservoirs or layers containing surfactant material) and because these films or layers likely have different properties than their solvent-spread equivalents, such systems need further reexamination under physiologically relevant concentrations and conditions.

The captive bubble tensiometer (CBT) allows for measurement of the surface-active properties of natural pulmonary surfactant and model surfactant mixtures under defined physiological conditions with respect to temperature, composition, and surface tension ranges (12, 24, 25, 27–29, 31–33). With this technique, suspensions of surfactant materials are adsorbed to the air-saline interface of the bubble. Surface tension changes during adsorption and compression-expansion of the bubble surface area can be monitored by bubble-shape analysis (28). This method allows for monitoring surface tension changes of films adsorbed to equilibrium surface tension, which are cycled between intermediate and low surface tensions as demonstrated occurring in the alveoli during the respiratory cycle (25, 30).

The advantage of the CBT over pulsating bubble surfacometers and surface balance methods is that leakage of film material over capillaries or barriers is avoided (34). The present study used the CBT to evaluate the surface-active properties of adsorbed films of DPPC with unsaturated PC or PG containing physiologically relevant amounts of SP-B (1 weight percent of the lipids). The study suggests that SP-B promotes rapid adsorption of both systems. SP-B also promotes selective DPPC adsorption and further refinement of the surface films with phospholipid mixtures containing the acidic but not the neutral lipid. These observations suggest a specific mode of interaction between this hydrophobic protein and certain surfactant lipids at the alveolar air-water interface.

**METHODS**

Materials. DPPC was purchased from Sigma (St. Louis, MO), and 1-palmitoyl-2-deoyl-sn-glycero-3-sn-glycero-1-phosphatidylcholine (POPC), 1-palmitoyl-2-[12-7-nitro-2,1,3-benzoxadiazol-4-yl]aminol]dodecanoyl-sn-glycero-3-phosphocholine (NBD-PC) were from Avanti Polar Lipids (Alabaster, AL). The lipids were checked for purity by silica gel thin-layer chromatography and used as received. Pulmonary SP-B was isolated from bovine lipid extract surfactant (BLES Pharmaceuticals, London, ON) with a modification of the LH-20/LH-60 method of Curstedt et al. (8) as discussed elsewhere (24). Column fractions were monitored by optical density at 280 nm and by dot blot analysis with an anti-bovine SP-B antibody received from D. J. A. Whitsett (Division of Pulmonary Biology, University of Cincinnati, Cincinnati, OH). The

process allowed for obtaining highly purified SP-B without other hydrophobic protein constituents such as SP-C. The pooled SP-B in chloroform-methanol (3:1) was analyzed with Tricine-sodium dodecyl sulfate-polyacrylamide electrophoresis (26) and electrospray ionization mass spectrometry (ESIMS) to detect the predominant monomeric versus dimeric forms present. Previous mass spectrometric analyses of this protein were able to detect only ionized protein at m/z 8,740, which does not distinguish between monomers and dimers (7, 20, 37).

Vesicle preparation. The lipids DPPC, POPC, and POPG were dissolved in chloroform-methanol (3:1) and mixed in desired proportions of DPPC-POPC or DPPC-POPG (7:3 wt/wt) with and without 1 (wt/wt) weight percent SP-B. The amounts of SP-B were estimated with a modification of the Lowry procedure as documented elsewhere (40, 41). The lipids and lipid-protein mixtures were dried under a stream of nitrogen in Teflon tubes. The dried mixtures were reconstituted in saline buffer (150 mM NaCl and 5 mM Tris·HCl, pH 6.9) with 2 mM CaCl₂ or 2 mM EDTA by sonicating the mixtures at 4°C with a microprobe sonifier (Branson Ultrasonics) with 5 bursts lasting 30 s each. The sonication was performed at low temperature to produce small unilamellar vesicles with minimal hydration to observe the maximal adsorption of the pure phospholipid mixtures as discussed by others (15, 39).

CBT. A custom-designed CBT (28) was used to evaluate the adsorption and dynamic and quasi-static compression-expansion cycles of the lipid-protein mixtures. Briefly, the CBT contained a glass chamber with an agar plug and a pressure-driven plunger to compress the fluid in the chamber. Typically, an air bubble of ~3–4 mm diameter was pulled into the fluid suspension containing the sample in the chamber, and the change in bubble shape with time or compression with the plunger was monitored and videorecorded with a light source and a camera. The shape of the air bubble or a change in the ratio of the minimal to maximal bubble diameter occurred depending on the surface tension of the air-solution interface of the bubble (28). The surface tension of the bubble was plotted as a function of the total area to give surface tension-area isotherms of the films formed at the solution-bubble interface (24).

Vesicular suspensions (1 mg/ml) of phospholipids with and without SP-B were studied in the CBT. After the CBT chamber was filled with the respective suspensions and the temperature was allowed to equilibrate at 37 ± 1°C, a small air bubble was introduced into the suspension. The change in bubble shape was recorded from 1 s after introduction of the bubble to 10 min to monitor adsorption of the materials to the air-saline interface of the bubble. After adsorption, the bubble chamber was sealed, and quasi-static compression-expansion of the bubble was performed by changing the bubble volume in steps (at a rate of ~5% of bubble volume per step) for five cycles.

In separate experiments (n = 3), dynamic compression-expansion of the bubble area was performed by cycling the bubble between 10 and 140% of the original area at a rate of 25 cycles/min. The bubble area was reduced until low tensions were attained (~10% of the original bubble surface area for the phospholipid mixtures and as low as 40% of the original area for the SP-B-containing mixtures) and was then expanded to near the original bubble area (90–140% of the original bubble surface area). Images documenting changes in the bubble area were recorded during each individual experiment, and bubble shapes were analyzed with customized software as previously described (24, 28).
All experiments were performed three or four times with individual freshly prepared samples. The methods of data analysis and experimental protocols are discussed elsewhere in a study (24) used to determine the properties of SP-C with similar lipid systems.

Fluorescence microscopy of films. Films of DPPC-POPG (7:3) plus 1 weight percent SP-B were solvent (chloroform-methanol 3:1 vol/vol) spread on a buffered saline subphase at 23°C in a Langmuir surface balance with epifluorescent microscopic attachments (19). The lipid-protein solutions contained 1 molar percent of the fluorescent phospholipid probe NBD-PC for visualization of structures in the film. The solvent-spread films were compressed and expanded in a manner and rate similar to those used to quasi-statically cycle the films in the CBT, and the images observed in such films were recorded on videotape. The surface technique and imaging of surfactant lipid-protein model films with this system, which are discussed in detail elsewhere (19, 20), have been used by others (1, 16, 17) as a general in vitro approach to examine the structures of pulmonary surfactant films.

Statistical methods. The results are presented as means ± SE from three independent studies. Statistical comparisons were conducted with the SPSS Base 9.0 Statistical Computer Program (SPSS, Chicago, IL). Unless otherwise stated, differences were compared by analysis of variance with Tukey’s post hoc test. Probability values of <0.05 were considered significant.

RESULTS

SP-B and SP-C are difficult to characterize with general biochemical methods due to their extreme hydrophobicity (2, 11, 13). As a result, in numerous previous studies on model lipid-SP-B systems, accurate information about the isolated protein and its conformation and structure was not provided (2, 13, 15). The integrated typical ESIMS spectrum of the pooled protein shown in Fig. 1 combines each of the individual ionic species from charges 9⁺ to 16⁺. Apparent monomers and dimers are detected with each positive charge. The integrated spectra show a major peak of 17,398 Da, which corresponds to dimeric SP-B. Small amounts of slightly higher-molecular-mass forms and a presumably truncated form at 17,155 Da were detected. Only small amounts of the protein were detected around 8,700 Da, corresponding to SP-B monomers. The peaks at ~8,700 Da and the additional peaks could arise, in part, from protein degradation that may occur even with the soft ionization procedure. These observations are consistent with the results of SDS-polyacrylamide gel electrophoresis where only dimeric forms of SP-B could be detected. The results also indicate that the isolated SP-B was essentially devoid of multiple trunc-
cated and methylated forms as previously reported by others (37), possibly due to differences in isolation procedure. The SP-B spectra show that a soft ionization spectrometry method such as ESIMS can reveal apparent intact dimeric forms of the protein compared with other mass spectrometry techniques that may not distinguish between monomers and dimers (7, 20).

Adsorption isotherms of DPPC-POPC and DPPC-POPG with and without SP-B in a captive bubble are shown in Fig. 2. The isotherms show that SP-B (1 weight percent) significantly enhanced the rate of adsorption of both phospholipid mixtures (P < 0.05). The protein promoted phospholipid adsorption to an equilibrium surface tension of ~20 mN/m within a few minutes. Similar adsorption rates were observed for the mixtures containing acidic (PG) or neutral (PC) phospholipid and SP-B. The results are consistent with previous findings (10, 23, 41) of the protein-enhancing adsorption rates of pulmonary surfactant phospholipids with other techniques and with studies (8, 13) where higher amounts of proteins (5–20 weight percent) were used.

Quasi-static compression-expansion isotherms of the equilibrium-adsorbed films of the phospholipid mixtures with and without SP-B are shown in Fig. 3. Figure 3A represents the mixtures containing the neutral phospholipids (DPPC-POPC 7:3 mol/mol), and Fig. 3B represents those containing acidic phospholipids (DPPC-POPG 7:3 mol/mol). The isotherms were constructed by averaging the first, second, and fourth compression and expansion cycles of three isotherms of the lipid-protein films. Isotherms of individual experiments from each system are shown in Fig. 3, insets. The averaged isotherms in Fig. 3A show that the films formed with the neutral lipid mixture without SP-B required relatively large area compressions of 70–80%
of the bubble area to reach minimal surface tension (Fig. 3A, top), whereas 50–60% area compressions were needed in the presence of the protein (Fig. 3A, bottom). With the phospholipid sample, an initial region of high compressibility, possibly indicating squeeze-out of fluid lipids, was observed around 30 mN/m, whereas a short but distinct plateau was noted near 20 mN/m with the protein-containing sample during compression. Even larger compression ratios of 80% were observed with the acidic phospholipids in the absence of protein, and surface tension did not reach near zero (Fig. 3B, top). In contrast, films of the mixtures of acidic lipids with SP-B required only ~30% area compression to reach a low surface tension near 0 mN/m, and smaller hysteresis in the isotherms was evident compared with either the pure lipid systems or the DPPC-POPC mixture containing protein (Fig. 3B, bottom). The results presented here showed that the interaction of SP-B with the acidic phospholipids resulted in the formation of films at the air-saline interface, which required less compression to achieve low surface tensions than the corresponding neutral mixture.

Because they start at different initial surface tensions, the surface area reductions indicated above are not directly comparable. Therefore, to obtain a valid assessment of surface activity, the percent area reductions required to achieve a low surface tension (<1 mN/m) from the equilibrium surface tension of 20 mN/m were determined for each mixture. Figure 4 displays the percentages of bubble area compression required for the phospholipid films without and with SP-B to generate a surface tension below 1 mN/m from the near-equilibrium value of 20 mN/m during quasi-static cycling. Figure 4A is for DPPC-POPC, and Fig. 4B is for DPPC-POPG. With the POPC system, the surface area reduction required fell from 35 to 20% by the fourth cycle. Surprisingly, SP-B slightly increased the surface area reduction required to reach a low tension in the neutral phospholipid system, although this was not significant. No improvement was observed with the acidic phospholipid system lacking protein during the four cycles. With SP-B present, <40% surface area reduction was required to achieve the surface tension to near 0 mN/m during the initial compression. By the third cycle, the area reduction required to attain a low tension with SP-B fell to 17 ± 4%. There was no further change in the fourth cycle. The effect of SP-B on the surface area reduction of DPPC-POPG films during the fourth compression was highly significant (P < 0.01). Previous studies (28, 32) have demonstrated that films of pure DPPC can achieve a surface tension near zero with a surface area reduction of ~15%. The small area reduction observed here with DPPC-POPG-SP-B mixtures indicates that in the third and fourth cycles, the films are highly enriched in or consist of almost pure DPPC.

Additional studies involving dynamic compression-expansion isotherms of adsorbed films were conducted with DPPC-POPC-SP-B (Fig. 5A) and DPPC-POPG-SP-B (Fig. 5B). The adsorbed films were cycled at a rate of 25 cycles/min, and in each cycle, the film area was changed between 10 and 140% of original area. Under these conditions, the films were compressed slightly beyond the point where low surface tensions are achieved, a situation known as film overcompression (24, 29). The data were averaged from three experiments, and only isotherms for cycles 1, 4, 10, and 21 are shown for clarity. Poor surface tension reductions were observed in the absence of SP-B (data not shown). With the neutral phospholipid-SP-B mixture, compressions of 40–60% were required to achieve a low tension during the initial compressions, and surface tension increased to over 50 mN/m during the first expansion. This was due in part to high compressibility (large area reduction needed to reduce surface tension) once the surface tension fell below 10 mN/m. Surface tension rose to above 50 mN/m during the initial expansion and 65 mN/m for the fourth and subsequent cycles. Surface area reductions required to attain a low tension remained around 50–60% of the original bubble area.

Lower compression ratios were required to achieve a low tension with the acidic phospholipid system (Fig. 5B). Surface tension rose to 35 mN/m during the first expansion and remained below 60 mN/m with the 21st cycle. Bubble area reductions of 30–40% were required to attain a low tension during the 21st compression. As a result of the smaller bubble area reductions required to produce a low surface tension and the slower increase in surface tension during expansion, the hyster-
esis loops observed with the acidic phospholipid system (Fig. 5B) were considerably smaller than those generated with the neutral system (Fig. 5A). The surface area reductions needed to reduce surface tension to near zero from the equilibrium value of 20 mN/m are depicted in Fig. 5C. With both systems, the surface area reductions required decreased progressively during dynamic cycling, indicating surface refining. The percentage area reduction required was lower with the acidic system throughout dynamic cycling, but this did not attain significance (P > 0.05; n = 3 experiments). These observations make it apparent that a major difference between the two systems was the higher surface tension achieved with the neutral phospholipid-containing system during the expansion phase.

Although it has been reported that calcium can enhance adsorption of phospholipid-SP-B mixtures and promote interactions between SP-B and acidic surfactant lipids, the role of this cation has not been clarified (11, 15, 22, 41). Therefore, studies with the acidic phospholipid system were conducted in the absence of calcium and in the presence of 2 mM EDTA to chelate any extraneous calcium arising from the water or solutes used in buffer preparation. Initial surface tensions during adsorption (Fig. 6A) were higher than those observed in the presence of the cation, and adsorption was more variable. Surface tension with the acidic lipid system fell to near 30 mN/m in 10 min in both the presence and absence of SP-B. The final surface tensions at 10 min were similar to each other. The final surface tensions were higher than the ~20 mN/m observed with SP-B-containing acidic samples in the presence of calcium.

The surface area reductions required to attain a low tension from equilibrium in the absence of calcium are shown in Fig. 6B. In the absence of SP-B, surface area reductions of 50% were initially required, but this decreased progressively to 30% by the fourth quasi-static cycle. Although not significant, considerably lower bubble area reductions were required in the presence of protein, with an area reduction of 30 ± 7% being required for the initial compression and only 17 ± 3% being needed by the third compression. The results reveal that in the absence of calcium, the POPG-containing system shows improved but not significantly different surface activity without SP-B, whereas overall surface properties similar to those with calcium are observed in the presence of the protein.

To obtain further information on the interactions between DPPC, POPG, and SP-B during surface area reduction and expansion, solvent-spread DPPC-POPG-SP-B films were examined during quasi-static cycling at room temperature (Fig. 7). The films were observed

![Fig. 5. Dynamic compression-expansion isotherms of DPPC-POPC-SP-B (A)- and DPPC-POPG-SP-B (B)-adsorbed films. Samples were prepared as described in Fig. 2. Films were cycled at a rate of 25 cycles/min between a bubble area of 140–10%. Initial bubble area (100%) was used to calculate bubble area of films before compression. The 1st (○), 4th (●), 10th (▲), and 21st (▼) cycles are depicted. Arrows, compression phase of isotherms. C: percent area reductions required to achieve surface tension < 1 mN/m from equilibrium surface tension of 20 mN/m. Open bars, neutral phospholipid-SP-B samples; hatched bars, acidic phospholipid-SP-B samples.](http://ajplung.physiology.org/)

![Fig. 6. A: adsorption isotherms for DPPC-POPG (7:3) with (■) and without (○) SP-B in absence of calcium and with 2 mM EDTA. B: percent bubble area reductions required to attain a surface tension of 1 mN/m from equilibrium (20 mN/m) with POPG system with (solid bars) and without (open bars) SP-B in absence of calcium.](http://ajplung.physiology.org/)
on a surface balance with an epifluorescent microscope attachment (19), and the images observed from the fluorescence of small amounts of fluorescent NBD-PC (1 molar percent of phospholipid) were included in the films. Condensed domains (Fig. 7, B–F, black areas) of phospholipids appear in such films on compression, and their relative area increases as surface tension declines. No distinct phase-separated isolated domains of fluid phospholipids (Fig. 7, bright areas) could be observed at any surface tension. Although the films appear highly condensed (Fig. 7, large fraction of the black areas) at surface tensions near equilibrium (Fig. 7, E and F), small connected fluid (fluorescent) regions remain present as a mesh surrounding the condensed domains. This result is in agreement with recent fluorescent studies (16–18, 20) with palmitic acid-SP-B and DPPG-SP-B systems where it was observed that the fluorescently labeled protein remained present in the continuous fluid-phase network.

**DISCUSSION**

Pulmonary SP-B and SP-C are difficult to characterize and quantify due to their extreme hydrophobicity (13). Most previous studies (2, 13, 15) on surface activity of the hydrophobic proteins did not define the exact structure and form of these isolated proteins, leading to some difficulty in systematic comparison of results from such studies. For example, some studies (6, 24) indicate that the dimeric and deacylated forms of SP-C have different surface activities and interactions with surfactant lipids compared with their native mono-
meric form. Others (37) have shown that the commonly used isolation procedures for SP-B can give rise to truncated and modified forms depending on the solvents used, and such altered forms of proteins cannot be easily detected by polyacrylamide gel electrophoresis. Also, various methylated and truncated forms of SP-C have been detected recently with nanoelectrospray mass spectrometry (9). These previous studies suggest that unambiguous detection of the exact form of the isolated hydrophobic surfactant proteins may be required to study their surface activity definitively. In addition, a highly purified form of the protein and one close to its native conformation in surfactant are required for such studies. Previously, mass spectrometry with matrix-assisted plasma desorption (7) or matrix-assisted laser desorption/ionization mass spectrometry (20) has been applied to SP-B, but these techniques could not distinguish between monomeric and dimeric forms of SP-B. The reason for this was that the above-mentioned analytic techniques use harsh conditions that can disrupt noncovalent and covalent bonds. Recently, application of the softer ionization methods involved in ESIMS has led to the detection of various forms of SP-C according to the different isolation procedures used (9). We therefore used ESIMS to analyze the purity and the conformation of the isolated SP-B used in this study (Fig. 1). The protein was found to be primarily in a dimeric form of 17-kDa molecular mass. Small amounts (10–15%) of monomeric forms of the protein of 8.7-kDa molecular mass and some other possibly truncated forms (<5%) were also present.

The adsorption of the phospholipid mixtures containing either neutral or acidic lipids was similarly enhanced by SP-B (Fig. 2). The phospholipids POPC and POPG were chosen for this study to minimize the effect of chain heterogeneity of phospholipids such as egg PG used in some previous studies. Also, physiological amounts of the protein (1 weight percent of phospholipid) corresponding to natural surfactant were used to observe the effect of SP-B on the lipids, in contrast to the higher amounts of protein used in some previous studies (16–18, 20, 36). Although the adsorption of phospholipids to the air-water interface is not well understood, it is influenced mainly by the fluidity of their chains and the hydration states of their head groups (15, 31, 39). Sonication of emulsions of the lipid-protein mixtures on ice at 4°C was used to keep hydration of the head groups minimal because minimally hydrated lipid vesicles adsorb faster than the fully hydrated form (15, 39). Previous studies (17, 18) have indicated that the positively charged amphiphilic helices of SP-B can interact with the head groups of surfactant acidic lipids. Other studies (4, 11, 15, 20) have suggested that this protein can perturb the packing of the phospholipids in bilayer vesicles and thereby increase their fluidity and ability to adsorb to an air-water interface. It appears possible from our study that both mechanisms of protein-induced enhanced adsorption of the phospholipids may be occurring. In the case of the neutral DPPC-POPC system, the perturbation of the chains, and in the case of DPPC-POPG, the PG head group dehydration by positive charges of SP-B, or a combination of both processes could occur.

In addition to affecting chain fluidity and head group hydration, SP-B could enhance adsorption by inducing lipid-packing defects into the vesicle structures (11). SP-B-induced packing defects produce leakage (lysis) and lipid mixing (semifusion) of lipid membranes. The close relationship between the dose-response curves for lipid mixing and phospholipid adsorption is consistent with a common mechanism, but more details are required (11). It was surprising to note that the adsorption rates of the neutral lipid with SP-B was similar to that of the acidic lipid because most previous studies (13, 41) have suggested that specific interactions between SP-B and PG are mainly responsible for enhanced adsorption of the phospholipids, although maximally hydrated systems and calcium were used in those studies.

A previous study (28) has shown that DPPC films require minimal surface area compression (∼15%) from equilibrium to reach a surface tension near 0 mN/m. Examination of such films with various techniques reveals very low compressibility below equilibrium surface tension (12, 20, 28). These studies indicate that relatively small area compressions are sufficient to order DPPC molecules into the highly ordered gel or condensed phase in films. In contrast, unsaturated phospholipids show high compressibility around the equilibrium surface tension and cannot be compressed to a low tension at physiological temperatures. Two mechanisms have been proposed to account for the surface monolayer enrichment in DPPC required for surfactant lipids to achieve a low tension during compression, selective DPPC adsorption, and preferential fluid phospholipid squeeze-out (15).

At the equilibrium surface tension of ∼20 mN/m, DPPC molecules possess an average molecular surface area of ∼42.5 Å², whereas POPC and POPG have average molecular areas of ∼50 Å² (15). With the assumption that the compositions of the initial adsorbed films are identical to the bulk dispersions (70% DPPC), it was calculated that DPPC molecules occupy 66.5% [((0.7 × 42.5)/((0.7 × 42.5) + (0.3 × 50))] of the surface area. POPC or POPG would occupy the remaining 33.5%. With ideal preferential squeeze-out (which is considered unlikely), a minimum area reduction of 33.5% would be required to exclude the fluid POPC or POPG. The remaining DPPC molecules would have to be compressed by 10.0% (66.5 × 0.15) to reduce surface tension from equilibrium to near zero. Thus ideal preferential squeeze-out would require a minimum of 43.5% (33.5% + 10.0%) area reduction to achieve low tensions during the first compression. Less than 40% area reduction from equilibrium was required to reach a low tension with the POPG samples containing SP-B in either the presence (Fig. 3B) or absence (Fig. 6B) of calcium. Comparison of these area compression values with the theoretical value, 43.5%, by paired t-tests revealed a significant difference for SP-B-containing samples (P < 0.01). This indicates that the ability to attain a low tension cannot be attributed to preferen-
tial squeeze-out of fluid POPG alone, although squeeze-out could contribute to the process. It should be noted the term “squeeze-out” indicates that the fluid lipid is excluded from the surface monolayer but does not imply that the unsaturated lipid moieties are no longer associated with the interfacial film (1, 3, 16, 24, 41).

Interestingly, it was consistently observed that <40% surface area reduction was required to achieve a low tension during the initial compression with the POPC-containing mixtures (Fig. 4A) with and without SP-B. However, it should be recalled that the protein-free samples were compressed from an initial surface tension of ~40 mN/m so that some enrichment due to squeeze-out could already have occurred at 20 mN/m. The low compression ratios observed with the SP-B-containing neutral mixture is also consistent with some selective adsorption of DPPC. The progressive improvement in surface activity observed with further quasi-static cycles could result from further partial selective DPPC adsorption during expansion, preferential exclusion of fluid POPC during compression, or a combination of these processes. These latter compressions involve total surface area reductions of ~50 (phospholipids alone) and ~35% (phospholipids and SP-B; Fig. 3A).

The rapid decline in required area reduction evident in subsequent compression-expansion cycles with the acidic phospholipid system could be explained by further enrichment in DPPC arising from selective adsorption during expansion, fluid lipid squeeze-out, or both in the presence of SP-B. These latter compressions initiate from around 30 mN/m. Adsorption of surfactant lipids can occur only at surface tensions above the equilibrium value of 20 mN/m. This would limit the amount of lipid that can insert into the surface film during expansion and suggests that preferential squeeze-out occurs during compression.

In the absence of calcium, the POPG system without SP-B exhibited superior adsorption than this system with the cation but did not reach the equilibrium surface tension in the presence or absence of protein with this minimally hydrated system (Fig. 6A). On compression, the POPG system lacking SP-B showed improved surface activity relative to the calcium-containing systems. The absence of calcium did not affect the surface activity of the DPPC-POPC-SP-B system. Again, <40% compression was required during the initial compressions, consistent with selective DPPC adsorption. The ability of SP-B to promote selective DPPC adsorption can also be concluded from previous captive bubble and pulsating bubble surfactometer studies (24, 32, 33, 41), although in some of those reports, the experimental observations were attributed to fluid lipid squeeze-out.

We interpret our quasi-static and dynamic cycling results as indicating a combination of selective DPPC adsorption and fluid lipid squeeze-out. A previous study (3) conducted with monolayers spread on the Langmuir-Willhemy balance was not able to demonstrate selective squeeze-out of fluid components. The difference from this earlier study can be explained in part by our use of the apparently leak-free CBT and also by the use of adsorbed rather than spread films. A recent study (31) on the structure of adsorbed surfactant films in captive bubbles with electron microscopy has indicated the existence of bilayer and multilayer structures associated with the air-bubble surface. Other CBT studies (24, 32) have suggested that such multilayers may act as surfactant reservoirs that can replenish the surface films with highly surface-active components of surfactant and that SP-C is involved in this process (i.e., DPPC). Recently, a structural study (1) of such films with atomic force microscopy has indicated that SP-C has a direct role in forming such multilayers at the air-water interface. The observation that the PG mixture was considerably more surface active than the neutral system suggests that SP-B may also have some role in forming such layers with the acidic phospholipid systems studied here. SP-B has been proposed to insert phospholipids from vesicles into a preformed monolayer by promoting lipid mixing between these layers (22), and this hydrophobic protein can also induce hemifusion of bilayers (4). The processes indicated above are enhanced by the presence of acidic phospholipids in the bilayer systems and require cations such as calcium. These studies suggested that SP-B has a direct role in the exchange and interactions of lipids between multilayers, resulting in DPPC enrichment, and this role is distinct from that of SP-C. Moreover, SP-B is more efficient than SP-C in some processes (22, 24). In the present study, the high enrichment of the surface films with DPPC in the DPPC-POPG-SP-B systems (Figs. 3 and 4) and the minimal hysteresis observed during dynamic cycling (Fig. 5) suggest a role for the protein in such bilayer-monolayer interactions. In the acidic lipid-containing system, SP-B may interact with PG phospholipids and limit their transfer or adsorption to the surface film, thereby allowing such films to be enriched by DPPC. In dynamic cycling experiments, the minimal hysteresis and lack of any plateau during the compression part of the isotherm (indicative of squeeze-out of the fluid PG component from the films) suggests that SP-B may prevent PG molecules from reabsorbing back into the films from the collapse phase during film expansion. A rapid fusion of the vesicles to the surface monolayers and lipid mixing between the monolayer and bilayer vesicles may also be induced by SP-B (4). For such processes to occur rapidly, the monolayer on the bubble must be in contact with an underlying layer or reservoir, and SP-B may act in-between these layered structures.

In comparison with a previous study (24) on DPPC-PG plus SP-C, the lower area reductions required to reach low surface tension from equilibrium in the SP-B system suggests that this protein is more efficient in the surface refining of films of surfactant, whereas SP-C is more effective at promoting respreading from the collapse phase. Thus, in keeping with previous reports, the hydrophobic proteins may have distinctly different roles in maintaining (SP-C) (30) and the selective exchange of (SP-B) lipids in a bilayer-monolayer system (22, 24, 36, 38). Although some
complex ionic interactions may be involved in the lipid-SP-B interactions, it appears the refining film process does not absolutely require the presence of calcium (Fig. 6).

The fluorescence images of the DPPC-DPPG-SP-B monolayer film shown in Fig. 7 indicate that a fluid-to-condensed phase transition occurs in the films, with increasing packing density and lower surface tension. Experimental limitations related to the small monolayer compression ratio available and possible film leakage onto and around the compression barrier with the fluorescence surface balance (19) used to study these planar films did not allow us to compress the films far beyond the equilibrium surface tension while visually observing the film air-water interface (Fig. 7). Because fluorescence monolayer studies have indicated that SP-B and SP-C peptides can form a connected fluid-phase network with other DPPC-acidic lipid films (16–18) and have shown that these proteins remain dispersed in the fluid phase of DPPC films (20), it appears reasonable to propose from our results that SP-B remains dispersed in the fluid (Fig. 7, fluorescent areas) regions of the DPPC-POPG films below equilibrium tension. If fluid phospholipid POPG is squeezed out of such monolayers, this probably occurs continuously from the fluid fluorescent regions of such films.

A recent study (16) with dipalmitoyl PG-SP-B planar films suggests that high amounts of SP-B (10 weight percent) in such films induces reversible buckling or folding of the films, with an increase in surface packing only in the presence of PG. This was not seen in films of SP-B and the neutral lipid DPPC (20). These buckled regions may correspond to SP-B-induced separate PG-enriched phase domains as concluded from other studies (20, 36, 41). The buckled structures (bilayer or multilayer) can remain in close association with the surface films and therefore could be reversibly adsorbed back onto the films on expansion (16). The study suggests that SP-B may thus prevent loss of some of the surface-active components from the air-water interface and supports our concept of multilayer structures in the bubble films. Also, the presence of SP-B in or near the surface monolayers during dynamic cycling can allow for rapid respreading of surface-active lipid-DPPC after film compression to a low surface tension. However, the case, a cautionary note is warranted in comparing data from solvent-spread planar films with adsorbed captive bubble films because the latter system may have a completely different lipid-protein arrangements than those in planar films studied. Also, the composition of the adsorbed surface films on the bubble may be quite different from that of solvent-spread films of the same mixtures, although complementary studies (2, 15, 38) with different techniques indicated that lipid-protein interactions similar to those in pulmonary surfactant occur at the alveolar air-fluid interface.

A number of previous surface tension-area studies (2, 13, 36, 41) on solvent-spread DPPC-POPG films containing SP-B have suggested that the protein may promote phase separation of the acidic lipid into distinct domains, possibly as a result of interactions between the acidic lipid and the positively charged amphipathic helices of the protein. Such phase-separated lipid-protein domains would be more readily squeezed out of the films on compression, thereby enriching the (film) surface monolayer with DPPC (18, 41). No evidence for isolated phase-separated PG-rich domains was apparent in the present study (Fig. 7), although it is possible such domains could be present but be below the detection limits of optical microscopy.

In conclusion, this study examined potential surfactant phospholipid-dimeric SP-B protein interactions under physiological conditions at the air-alveolar fluid interface. The results show that SP-B enhances adsorption and formation of highly surface-active films of surfactant phospholipid through selective adsorption and/or progressive squeeze-out at an air-water interface. These processes probably involve both ionic and hydrophobic interactions of SP-B with the acidic lipid PG component of the surfactant. It appears that calcium is not absolutely required for these processes and that the cation may more likely be linked with the hydration states of the lipids. Structural features of comparable planar films of the lipid-protein system indicate that highly condensed (DPPC-rich) films and a fluid acidic phospholipid mesh coexist near the equilibrium surface tension. Also, films formed from the acidic lipid-SP-B systems show optimal surface tension-film area characteristics comparable to native surfactant, that is, 1) rapid film formation at an air-water interface, 2) minimal area compression to reach low surface tension, and 3) rapid respreading after film compression. Such systems may have potential in artificial surfactant replacement therapy.

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