Articles

Dynamic Surface Properties of Pulmonary Surfactant Proteins SP-B and SP-C and Their Mixtures with Dipalmitoylphosphatidylcholine†

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ABSTRACT: Dynamic cyclic surface pressure (π)-area measurements were performed on surfactant proteins SP-B and SP-C in pure and binary spread films with dipalmitoylphosphatidylcholine (DPPC). When the films of pure SP-B and SP-C were compressed beyond their collapse points (about 40 mN m^{-1}), no appreciable irreversible loss of material occurred and the successive compression isotherms were reproducible. A similar reversible collapse for the proteins was observed when their binary films with DPPC were compressed up to high surface pressures ($\pi \approx 65$ mN m^{-1}), which did not surpass the collapse for DPPC (about 72 mN m^{-1}). In this case, SP-B, squeezed out at 50 mN m^{-1} during compression of the SP-B/DPPC monolayers that contained ≥10 weight % protein, reinserted in the films during their subsequent expansion. Likewise, SP-C−DPPC complexes were reversibly excluded at $\pi \approx 55$ mN m^{-1} from the SP-C/DPPC films that contained ≥5 weight % protein. Dynamic compression of the mixed protein−lipid films beyond the collapse pressure of DPPC showed that SP-B and SP-C improved the respreading of DPPC in a concentration dependent manner. SP-B was more effective in promoting the respreading of DPPC than was SP-C, as indicated by the collapse plateau length ratio criterion. The results from this study suggest a possible interfacial role for SP-B and SP-C in lipid replenishment at the alveolar−air interface, through enhancement of the respreading of DPPC collapse phases (SP-B and SP-C) or through reversible removal of phospholipid (SP-C) during dynamic cyclic compression−expansion of the alveolar surface.

Pulmonary surfactant, through its surface-active properties, plays an important role in the mechanics of the lung (Clements, 1961; Goerke, 1989). Rapid adsorption from the hypophase into the alveolar interface, lowering the surface tension to near zero during maximum compression, and respreading into the interface after dynamic compression past collapse are considered to be essential for the function of pulmonary surfactant *in vivo* (Clements, 1977; Notter et al., 1980a,b; Notter & Morrow, 1975; Obladen et al., 1979; Keough, 1992). It is well recognized that dipalmitoylphosphatidylcholine (DPPC),[1] the major phospholipid component of lung surfactant, alone does not have all of the surface properties necessary to fulfil all the requirements of surfactant *in situ*. While DPPC can sustain high surface pressures during compression at the air−water interface, it exhibits slow interfacial adsorption from aqueous dispersions at temperatures below the gel-to-liquid crystal transition temperature of 41 °C (Notter et al., 1982a) and poor respreading from films compressed beyond collapse points (Snik et al., 1978; Notter et al., 1980a,b; Keough et al., 1983). A large number of studies, concerned with preparation of substitutes of lung surfactant for replacement therapy, have been directed at the effects of additional pulmonary surfactant components, such as unsaturated phospholipids, phosphatidylglycerols, and cholesterol, on the adsorption rate (Obladen et al., 1979; Notter et al., 1983) and on the respreadability of DPPC (Notter et al., 1980a,b; Fontanges et al., 1984; Fleming et al., 1983; Fleming & Keough, 1988). The hydrophobic pulmonary surfactant-associated proteins, SP-B and SP-C, enhance the rate of adsorption of phospholipids from vesicles in the subphase into the air−water interface. Surface adsorption measurements under equilibrium (Suzuki, 1982; Hawgood et al., 1987; Shiffer et al., 1988; Oosterlaken-Dijksterhuis et al., 1991) and dynamic conditions (Takahashi & Fujiwara, 1986; Yu & Possmayer, 1986, 1990; Curstedt et al., 1987; Mathialagan & Possmayer, 1990; Takahashi et al., 1990) have shown that some phospholipid mixtures reconstituted with the hydrophobic pulmonary surfactant proteins mimic the surface activity of natural pulmonary surfactant.

Our previous studies showed that the hydrophobic pulmonary surfactant proteins, when present in monolayers of DPPC up to about 16 wt %, did not interfere with the ability of DPPC films to achieve maximum surface pressure of about 72 mN m^{-1} when they were quasistatically compressed (Taneva & Keough, 1994a,b). Results indicated that at small

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[1] Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]-(sodium salt); SP-B, pulmonary surfactant-associated protein (M_r 17 400); SP-C, pulmonary surfactant-associated protein (M_r 4186)

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initial concentrations of SP-B and SP-C in the films (less than about 10 and 5 wt %, respectively) the proteins remained in their binary films with DPPC during compression to high surface pressures near 72 mN m^{-1} where collapse phases composed of lipid plus protein likely were formed. This finding suggested that the proteins incorporated into the collapse phase formed near 72 mN m^{-1} might have the possibility of altering the mechanism of collapse of DPPC and might yield better respreadability of the lipid. Previous measurements in our laboratory also suggested that the hydrophobic pulmonary surfactant proteins, enriched in SP-C, enhanced the respreading rate of phospholipid monolayers containing DPPC (Pérez-Gil et al., 1992). In the present study, we have investigated the influence of SP-B and SP-C on two surface characteristics of the monolayers of DPPC: the hysteresis occurring in the cyclic surface pressure–area curves and the respreadability after dynamic compression past monolayer collapse. SP-B, $M_r = 17\,400$, is a disulfide-linked dimer of 79 amino acid residue monomers which contains regions of amphiphatic α-helix and has a net positive charge at physiological pH (Curstedt et al., 1988; Takahashi et al., 1990). SP-C is an extremely hydrophobic 35-residue protein which has two palmitoyl groups bound through thiol esters of cysteines at positions 5 and 6 of its sequence. It has a 23-residue C-terminal α-helical portion and also has a net positive charge associated with residues near its N-terminal (Curstedt et al., 1990).

EXPERIMENTAL PROCEDURES

Materials. Dipalmitoylphosphatidylcholine (DPPC) was obtained from Sigma Chemical Co. (St. Louis, MO), and dipalmitoylphosphatidylglycerol (sodium salt) (DPPG) was from Avanti Polar Lipids Inc. (Alabaster, AL). The phospholipids were analyzed for purity by thin-layer chromatography and were used as received. Cholesterol was 99% Sigma Grade (Sigma Chemical Co., St. Louis, MO). Sodium chloride and calcium chloride, reagent grade, were obtained from Fisher Scientific (Ottawa, ON). Water was deionized and doubly-distilled in glass, the second distillation being from a dilute potassium permanganate solution.

Purification of SP-B and SP-C. Pulmonary surfactant proteins SP-B and SP-C were prepared from porcine lavage fluid as described previously (Taneva & Keough, 1994a). Gel exclusion chromatography on Sephadex LH-60 [chloroform/methanol 1:1 (v/v) containing 1% (by volume) of 0.1 M HCl as opposed to 5% of 0.1 M HCl in the previous paper (Taneva & Keough, 1994a)] was used for isolation and purification of the hydrophobic proteins from the lipid extract of surfactant. On SDS–polyacrilamide gel electrophoresis (16% gels) (Laemmli, 1970) under nonreducing conditions, SP-B yielded a major band at about 18 kDa and a minor one at about 29 kDa. SP-C showed one band at about 5 kDa. The gels were stained with silver stain (Daiichi Pure Chemicals Co., Ltd., Tokyo). The estimation of protein was done by the fluorescamine assay (Udenfriend et al., 1972) which gave results similar to those obtained by quantitative amino acid analysis (Sarin et al., 1990). Analysis of phosphorus in the protein preparations (Bartlett, 1959; Keough & Kariel, 1987) showed less than 0.5 mol of phospholipid per mol of SP-B (dimer) and less than 0.05 mol of phospholipid per mol of SP-C (monomer).

Surface Pressure Measurements. Surface pressure measurements were performed on a Langmuir trough, Applied Imaging (Dukesway Team Valley, Gateshead, Tyne and Wear, England) which employed a continuous teflon ribbon barrier. The use of the ribbon barrier allowed minimization of film leakage and hence reliable measurements in the region of the high surface pressures. Surface tension was measured by the Wilhelmy plate method (using a platinum plate roughened by scratching with emery paper). Known amounts of DPPC and SP-B, or SP-C, were spread from a common solvent (chloroform/methanol, 3:1, v/v) into the air–water interface to form mixed monolayers. The subphase was 150 mM NaCl plus 2 mM $CaCl_2$, a concentration chosen to reflect that in the extracelluar 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 the surface pressure measurements. The temperature was 22–24 °C.

Dynamic cyclic surface pressure–area measurements were performed under various conditions, such as initial spreading pressure, maximum surface pressure attained in the films during compression, time for compression–expansion cycle, and relative change in the film area. These conditions are specified further in the text below. In all experiments the films were continuously compressed and expanded four times. We note that the experimental conditions in this study, e.g., cycle frequency, ratio between maximum and minimum area, T °C, did not necessarily correspond to the dynamic conditions in lung *in vivo*. However, the experiments here allow an understanding of the effect of the hydrophobic pulmonary surfactant proteins on the dynamic surface properties of DPPC, which usually are assessed from *in vitro* measurements under conditions similar to these used here. According to the terminology of Notter et al. (1980a), we used two initial spreading conditions: (i) "surface dilute" conditions, where the amounts of the initially spread lipid, or lipid–protein mixtures, were small and the initial surface pressures after spreading were low (below 5 mN m^{-1}); and (ii) "surface excess" conditions, where the amount of surface active material initially spread was in excess to the amount required for monolayer surface coverage at the start of dynamic compression. In this case the area of the mixed monolayers was expressed as a trough area, A, whereas in the case of initial "dilute" concentrations, it was defined as mean area per "residue", A_{mean}, where "residue" denoted a phospholipid molecule or a protein amino acid residue. The initial concentrations of protein in lipid–protein monolayers were expressed as weight % of protein and as "residue" fractions of the protein amino acid residues, $X_r = N_r/(N_r + N_l)$, where N_r and N_l were the numbers of the amino acid residues of protein and lipid molecules initially spread.

RESULTS AND DISCUSSION

Dynamic Cyclic Surface Pressure–Area Properties of Monolayers of SP-B and SP-C. Monolayers of either SP-B or SP-C were formed at an initial surface pressure of 0 mN m^{-1}. Four compression–expansion isotherms were measured at a cycling time of 230 s each. The relative change in the film area was 80%. The results, plotted in Figure 1, showed that during compression of the films of SP-B and SP-C, maximum surface pressures of about 46–47 mN m^{-1} were attained. After the fifth compression of the films to minimum area, barrier movement was stopped, and relaxation in the surface pressure was monitored. The protein films relaxed to constant values of π of about 37–38 mN m^{-1},

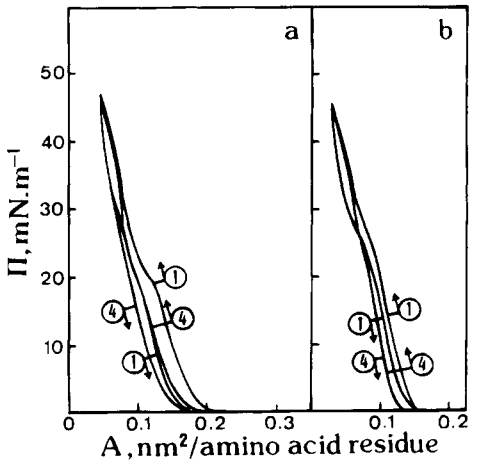

FIGURE 1: Dynamic cyclic surface pressure–area isotherms for films of SP-B (a) and SP-C (b). Relative change in monolayer area was 80% and cycling time was 230 s. Cycles 1 and 4 are shown. The curves are representative sample of two measurements.

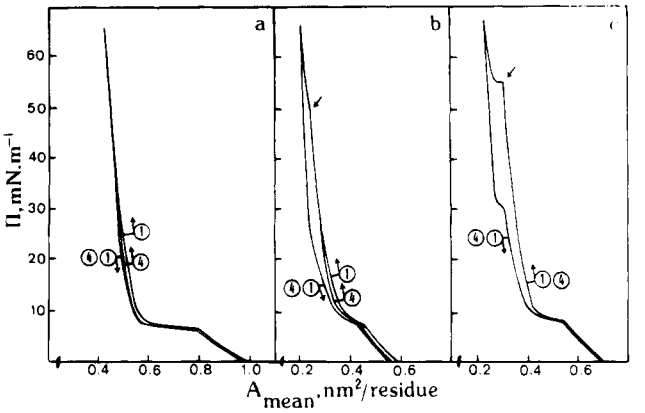

FIGURE 2: Dynamic compression–expansion isotherms of DPPC (a); DPPC plus 17 wt % SP-B, or $X_r = 0.57$ (b); DPPC plus 10 wt % SP-C, or $X_r = 0.41$ (c). Cycles 1 and 4 are shown. Arrows indicate surface pressures of squeeze-out of the proteins. Relative change in monolayer area was about 55% and cycling time was 130 s.

close to the collapse pressures of the films under quasi-static compression. The first compression–expansion isotherms for the films of SP-B (Figure 1a) and SP-C (Figure 1b) exhibited hysteresis in the whole range of surface pressures. The fourth compression–expansion isotherms for SP-B and SP-C films were slightly shifted to lower areas at surface pressures below 30 mN m^{-1}. However, at the higher pressures the first and fourth compression and expansion curves coincided for the films of SP-B and SP-C, and this observation suggested that the kinetic processes responsible for the hysteresis in the protein films were mostly reversible. Processes operating in protein films compressed beyond their collapse point may involve changes in the packing state in the monolayer (Lavigne et al., 1992), reversible displacement and reentry of segments of the molecules or irreversible desorption of protein molecules (McRitchie, 1981), or transition from a monolayer to bilayer state (Malcolm, 1973). An additional reason for the hysteresis may involve reversible adhesion of the hydrophobic proteins to the trough or Wilhelmy plate. The slight difference in the hysteresis properties of films of the two proteins possibly resulted from differences in the kinetics of their collapse or respreading in the surface.

Dynamic Interfacial Behavior of SP-B and SP-C in Binary Monolayers with DPPC. The aim of the cyclic compression–expansion surface pressure–area measurements presented in this section was to investigate the contribution of the hydrophobic pulmonary surfactant proteins to the hysteresis in the surface pressure observed in the films of DPPC. The relevance of the hysteresis in phospholipid films for lung surfactant function in vivo has been discussed (Galdston & Shah, 1967; Notter et al., 1982b). The large hysteresis observed in those studies and seen here in some films compressed beyond collapse is possibly most relevant to large excursions of lung volume in situ, particularly when they occur from low lung volume or low lung pressure. During tidal volume breathing, hysteresis in lung surface tension is small (Bachofen et al., 1970). Such small hysteresis has been found in surfactant films compressed over small volumes in captive bubble, especially when such films appeared not to be compressed beyond collapse (Schürch et al., 1992). The work here suggests that compression of films up to, but not beyond, the point of film collapse also results in very small amounts of hysteresis. Understanding film respreading in the presence or in the absence of hysteresis can help us derive fundamental information about the mechanisms that drive the spreading process. Both types of model systems may have physiological or pathophysiological counterparts.

A number of molecular events may determine the hysteresis seen in the surface pressure–area plots for monolayers at the air–water interface (Taneva, 1986): (i) exchange of surface active molecules between the surface and bulk liquid phases; (ii) rearrangement or reorientation of molecules in the monolayer plane; (iii) monolayer collapse and respreading of collapse phases; (iv) frictional drag between the monolayer and the liquid subphase, or Marangoni effect (Dimitrov et al., 1978). These processes lead to hysteresis in the surface pressure–area diagrams during monolayer cyclic compression–expansion when their characteristic times are in the time scale of, or longer than, the cycling frequency. The potential role of these processes for the hysteresis in the surface pressure–area curves for the alveolar monolayer has been analyzed (Mehta & Nagarajan, 1982). In this section we present results from cyclic surface pressure–area measurements on spread films of DPPC alone and DPPC plus hydrophobic protein, compressed to surface pressures which exceeded the collapse pressure of the protein but did not exceed the collapse plateau for DPPC. Therefore, the observed hysteresis would possibly be due to rearrangement and reorientation of the molecules in the monolayer and collapse of the protein component. Two series of experiments were performed. In the first one, the mixed films were spread at "surface dilute" concentrations, which generated initial surface pressure of less than 1 mN m^{-1}, and they were compressed to maximum surface pressure of about 65 mN m^{-1}. This pressure was higher than the collapse pressures of SP-B and SP-C defined as maximum pressures attained in the films of pure proteins under similar dynamic conditions (see Figure 1), and it was below the pressure corresponding to the onset of collapse of DPPC (about 65 mN m^{-1}). In films of DPPC, at $\pi > 65$ mN m^{-1} formation of collapse nuclei sets in (Snik et al., 1978). The cyclic pressure–area plots for the films of pure DPPC, shown in Figure 2a, exhibited small hysteresis, which may be attributed to relaxation processes (Snik et al., 1978; Taneva et al., 1979;

Notter et al., 1982b), surface rearrangements during phase transitions, or nonhomogeneous ejection of film molecules from the interface during dynamic compression of the monolayers (Notter et al., 1982b). The first and fourth dynamic isotherms for DPPC coincided in the region of high pressures, and this observation, in agreement with those observed previously (Notter et al., 1982b), suggested that mostly reversible processes were operating in the films under these experimental conditions.

Figure 2b,c shows the results for DPPC films which contained 17 wt % SP-B, or $X_r = 0.57$ (b) and 10 wt % SP-C, or $X_r = 0.41$ (c). These protein concentrations, higher than the amount of the hydrophobic protein usually reported for lung surfactant, were deliberately chosen to demonstrate the contribution of the collapse of the proteins to the hysteresis in the surface pressure in the mixed films. Our previous studies suggested that the hydrophobic proteins were squeezed out during compression of the DPPC/protein films which contained more than 10 wt % SP-B or more than 4 wt % SP-C (Taneva & Keough, 1994a,b). The calculations showed that nearly pure SP-B, possibly associated with 1−3 lipid molecules, was excluded, whereas SP-C appeared to remove about 8−10 lipid molecules during squeeze-out (Taneva & Keough, 1994a,b). Comparison between the results in Figure 2a and those in Figure 2b,c showed that the protein−lipid films exhibited greater hysteresis in the pressure−area loops at $\pi > 10$ mN m^{-1} than did the films of pure DPPC. The superimposition of the first and fourth compression−expansion cycles suggested that reversible processes, such as changes in the orientation and packing of the molecules in the monolayer and expulsion and reentry of the protein moiety, likely contributed to the hysteresis in the surface pressure in the mixed films.

From the results in Figure 2b,c it appeared that the proteins, excluded from the mixed films during compression, possibly remained associated with the lipid-enriched monolayer and reinserted into films during subsequent expansion. Such behavior is not apparent for all lipid−protein films. Under similar dynamic conditions, spread films of DPPC plus serum albumin showed that part of the protein was irreversibly lost from the monolayers during cyclic compression (Taneva et al., 1984). As mentioned above, the squeeze-out of the hydrophobic pulmonary surfactant proteins, particularly SP-C, appeared to be accompanied by squeeze-out of phospholipid. The reproducibility of the dynamic cyclic isotherms for SP-C/DPPC films (Figure 2c) suggested that SP-C, through its reversible exclusion and reentry, may provide a means for reversible removal of phospholipid from the films. As discussed later, under dynamic compression past collapse of DPPC, such a reversible removal of DPPC at the low pressures (50−55 mN m^{-1}) would prevent part of the phospholipid from irreversible collapse at the high pressures and may represent a mechanism for phospholipid replenishment of the monolayer.

In a second series of dynamic cyclic experiments, the monolayers of pure DPPC, or DPPC plus hydrophobic surfactant protein were compressed up to surface pressure of about 71 mN m^{-1}, just below the collapse plateau for DPPC (Figure 3). Each of the monolayers of DPPC alone and those of DPPC plus hydrophobic protein contained an equal amount of DPPC, corresponding to 0.97 nm^2 per molecule of DPPC at the start of dynamic compression. The maximum surface pressure chosen in these measurements

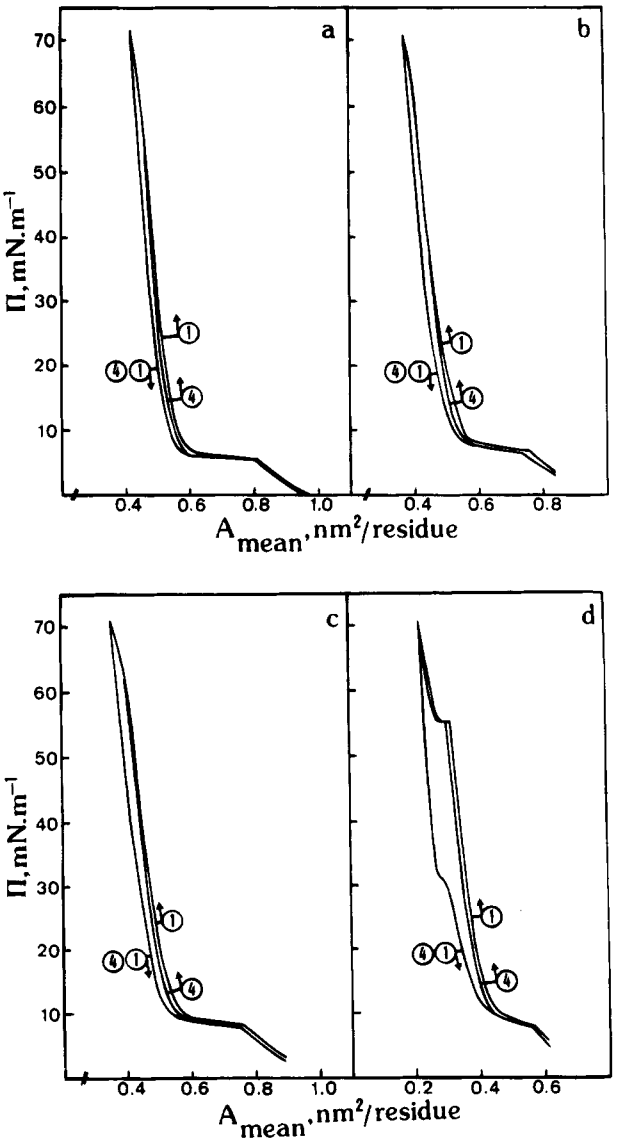

FIGURE 3: Cyclic compression−expansion isotherms of DPPC (a); DPPC plus 2.5 wt % SP-B, or $X_r = 0.15$ (b); DPPC plus 2.5 wt % SP-C, or $X_r = 14$ (c); DPPC plus 10 wt % SP-C, or $X_r = 0.41$ (d). Cycles 1 and 4 are shown. Relative change in monolayer area was about 60% and cycling time was 140 s.

was relevant to the lowest surface tensions (below 1 mN m^{-1}) evaluated for the pressure−volume loops of lung (Bachofen et al., 1987). Comparison of the results for DPPC monolayers, shown in Figures 2a and 3a, revealed that the compression of the films to the higher surface pressure resulted in a slightly greater hysteresis in the pressure−area cycles and a slight shift of the fourth compression isotherm toward lower areas. The effects were small, but nevertheless they suggested that, in addition to the reversible relaxation processes in the monolayers, gradual irreversible collapse of DPPC possibly commenced and contributed to the observed hysteresis when the films were compressed to 71 mN m^{-1}. The addition of 2.5 wt % of SP-B or SP-C showed almost no effect on the hysteresis properties of the films of DPPC (Figure 3b,c). At these concentrations neither SP-B nor SP-C seemed to be excluded from the DPPC films. It is worth noting that these low protein concentrations were relevant to the amounts of the hydrophobic proteins reported for surfactant lipid extracts (Yu et al., 1987). However, the exact amount of hydrophobic protein in the monolayer in

lung is unknown, and it is recognized that the ratio between the phospholipid and hydrophobic protein in different surfactant structures may vary (Hawgood et al., 1987). Different authors have suggested and used in model systems amounts of SP-B or SP-C well in excess of 2−3 wt % hydrophobic protein [e.g., Shiffer et al. (1988) and Oosterlaken-Dijksterhuis et al. (1991)]. Higher levels of SP-B or SP-C (e.g., 10 wt %) in DPPC films led to a greater hysteresis in the dynamic cyclic pressure−area isotherms (Figure 3d). At this concentration squeeze-out of the protein was detected at $\pi \approx 55$ mN m^{-1}, and therefore the greater hysteresis, compared to that seen in the SP-C/DPPC films of lower protein concentration (Figure 3c), may be attributed to the reversible displacement and reentry of SP-C associated with some DPPC.

In summary, during dynamic cyclic compression−expansion measurements where the maximum surface pressure was comparable to the surface pressure at the end of expiration in lung, though it was lower than the collapse plateau of DPPC, the films of DPPC containing low amounts of either SP-B or SP-C (e.g., 2.5 wt %) showed very small hysteresis, due to predominantly reversible relaxation processes in the films. Similar dynamic properties were seen in the films of pure DPPC. Greater hysteresis was observed in the films of DPPC plus 10 wt % SP-B or SP-C, where reversible exclusion and reentry of the proteins occurred.

Effect of the Hydrophobic Surfactant Proteins, SP-B and SP-C, on the Postcollapse Respreading of DPPC Monolayers. Dynamic surface pressure−area measurements on lipid and lipid−protein 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 hydrophobic protein contained an equal amount of DPPC corresponding to 0.97 nm^2 per molecule of DPPC at the start of compression. The initial spreading surface pressures were less than 5 mN m^{-1}. Cycling time was 230 s and relative change in the monolayer area was 80%. Figure 4a shows the first and fourth cyclic isotherms for the films of DPPC alone. The displacement of the fourth compression isotherm to low areas and the decrease in the collapse plateau length of successive cycles indicated poor respreading of DPPC molecules that had been squeezed out from the surface during monolayer collapse. As a measure of the extent of respreading of the films we used the collapse plateau length ratio criterion (Turcotte et al., 1977; Notter et al., 1980a,b). The collapse plateau length ratios for DPPC (Table 1), in agreement with other authors' data (Notter et al., 1980a; Fleming et al., 1983), demonstrated that considerable amount of the initially spread molecules of DPPC was irreversibly lost from the surface during the first compression past collapse. The collapse mechanism of DPPC, not well understood, may involve ejection of film molecules into subphase lamellae or formation of stable surface collapse structures (Turcotte et al., 1977; Snik et al., 1978). The respreading of DPPC from collapse phase may be important as a possible route for replenishment of lipid at the air−water interface in lung during the breathing cycle (Notter et al., 1975; Keough, 1992) and as a delivery route of surface active material during exogenous treatment of pulmonary surfactant insufficiency in vivo (Turcotte et al., 1977). It has been shown that some pulmonary surfactant components, such as unsaturated phospholipids (PGs and PCs) facilitate the respreading of DPPC from the collapse

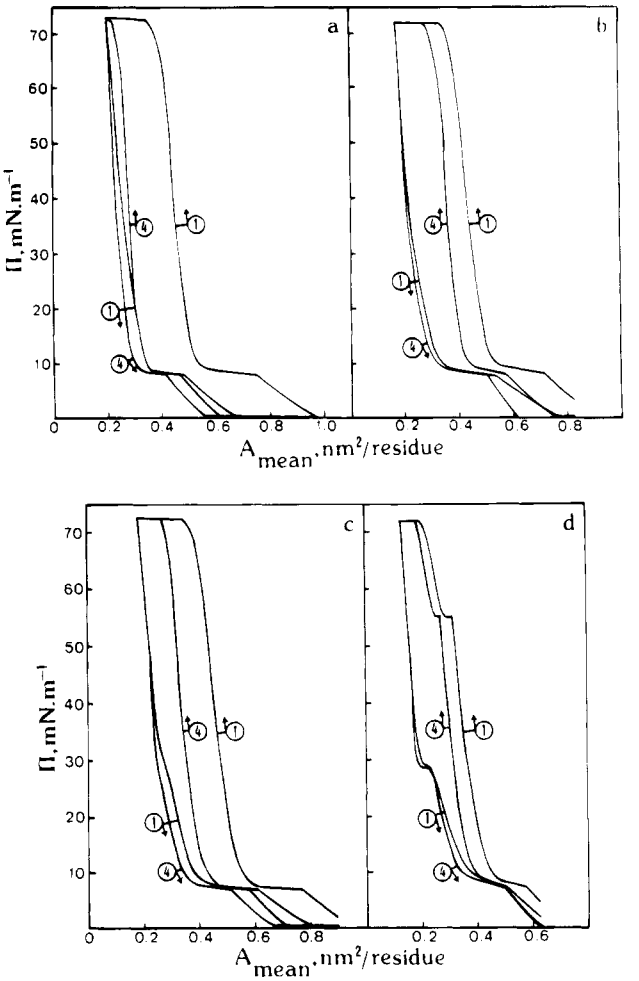

FIGURE 4: Dynamic compression−expansion behavior of DPPC (a); DPPC plus 2.5 wt % SP-B, or $X_r = 0.15$ (b); DPPC plus 2.5 wt % SP-C, or $X_r = 0.14$ (c); DPPC plus 10 wt % SP-C, or $X_r = 0.41$ (d). Cycles 1 and 4 are shown. Initial spreading concentrations in the films corresponded to 0.97 nm^2 per molecule of DPPC. Relative change in monolayer area was 80% and cycling time was 230 s.

phase (Notter et al., 1980a,b; Fleming et al., 1983), whereas others, e.g., cholesterol, essentially have no effect on respreading (Snik et al., 1978; Notter et al., 1980b).

Figure 4b,c shows results for the dynamic cycling of films of DPPC plus 2.5 wt % SP-B or SP-C, each compressed past the collapse of DPPC. The hydrophobic protein, SP-B (4b) or SP-C (4c), was not separately excluded from the monolayers during compression. Therefore, the collapse phases, formed at maximum compression, likely consisted of lipid−protein mixtures of compositions identical or very similar to the monolayer compositions at the start of compression. The length of the collapse plateau of the first cycle for the films of DPPC plus protein (Figure 4b,c) was longer than that for the films of pure DPPC (Figure 4a). Given the fact that each of the films in Figure 4a−c contained an equal amount of DPPC, this observation was consistent with the idea that SP-B and SP-C were not ejected from the mixed monolayers, and they were incorporated in the collapse phases formed at about 72 mN m^{-1}. Had SP-B and SP-C been selectively squeezed out of the monolayers, the lengths of collapse plateaux for the binary films would have approached that of a pure DPPC monolayer.

Figure 4d shows the behavior of films of DPPC plus 10 wt % SP-C, or $X_r = 0.41$ compressed beyond the collapse

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

monolayer	concentration of non-DPPC component			initial spreading surface pressure (mN m^{-1})	collapse plateau length ratios	
	wt %	mol %	X_r[b]		2/1	4/1
DPPC				0.2	0.54 ± 0.05[c]	0.34 ± 0.06
SP-B/DPPC	0.5	0.02	0.03	2.2	0.76	0.60
SP-B/DPPC	1.0	0.04	0.06	1.5	0.82	0.68
SP-B/DPPC	2.5	0.11	0.15	3.4	0.91 (0.37)[d]	0.78 (0.29)
SP-B/DPPC	10.0	0.47	0.43	6.5	0.84 ± 0.03	0.75 ± 0.03
SP-C/DPPC	0.5	0.09	0.03	1.9	0.56	0.35
SP-C/DPPC	1.0	0.18	0.06	2.1	0.63 ± 0.01	0.39
SP-C/DPPC	2.5	0.45	0.14	2.2	0.75 ± 0.03	0.56 ± 0.05
SP-C/DPPC	5.0	0.92	0.25	2.8	0.91	0.80
SP-C/DPPC	10.0	1.92	0.41	4.7	0.88 ± 0.05	0.77 ± 0.04
DPPG/DPPC	10.0	9.8		5.3	0.56	0.35
DPPG/DPPC	30.0	29.7		2.5	0.77	0.64
DPPG				1.9	0.83	0.61
cholesterol/DPPC	8.0	14.2		0.2	0.29 ± 0.03	0.08 ± 0.02

[a] Each monolayer contained an identical amount of DPPC, corresponding to 0.97 nm^2 per molecule of DPPC at the start of dynamic compression. In each experiment, for each successive cycle, maximum surface pressure of 72 ± 1 mN m^{-1} was attained at the end of compression and surface pressure of about 0 mN m^{-1} was reached at the end of expansion. [b] X_r is the fraction of amino acid residues of the protein in the mixed films. [c] The values represent mean ± SD (n = 3). [d] The values in parentheses represent results from experiments where surface pressure was >0 mN m^{-1} at the end of expansion (see Figure 6b).

pressure of DPPC. At this level SP-C was squeezed out from the binary monolayers during compression to π > 50 mN m^{-1}; about 20% of the initially spread DPPC was removed during exclusion of SP-C (Taneva & Keough, 1994b). The length of the collapse plateau of the first compression isotherm for SP-C/DPPC films at $\pi \approx 72$ mN m^{-1} (Figure 4d) was considerably shorter than that for DPPC alone (Figure 4a), consistent with removal of phospholipid at the lower pressure ($\pi \approx 55$ mN m^{-1}). We calculated that the phases excluded at π > 55 mN m^{-1} were enriched in SP-C (Taneva & Keough, 1994b); however, we could not reliably estimate whether some of the initially spread protein remained in the monolayer after the squeeze-out. It is possible that some SP-C was incorporated in the collapse phase formed at 72 mN m^{-1}. From the results in Figure 4d it appeared that the two excluded phases formed during compression of the films, one at 55 mN m^{-1}, enriched in the protein component, and one at 72 mN m^{-1}, enriched in DPPC, exhibited good respreading properties, so that the first and fourth compression isotherms at π > 55 mN m^{-1} were very close.

The collapse plateau length ratios for the cycling of monolayers in Figure 4a–d, summarized in Table 1, showed the superior respreading of the films of DPPC containing hydrophobic protein over those of DPPC alone. Values of the ratios for cycle 2/cycle 1 and cycle 4/cycle 1 for the lipid–protein films, compared to those for DPPC alone, revealed that SP-B and SP-C enhanced the respreading of DPPC in a concentration-dependent manner. The effect of SP-B on respreadability of DPPC seemed to plateau at about 2.5 wt % (X_r = 0.15) protein, whereas for SP-C this saturation point corresponded to 5.0 wt % protein (X_r = 0.25). The finding that the effect of 0.5 wt % SP-B was comparable to that of 2.5 wt % SP-C suggested a greater effectiveness of SP-B in improving the respreading of DPPC. This is in keeping with observations that lower concentrations of SP-B than SP-C were required for optimal adsorption of phospholipid mixtures from vesicles in the subphase into the interface (Yu & Possmayer, 1990).

SP-B and SP-C showed better facility of promoting the respreading of DPPC from collapse state than did bacterial PG (Notter et al., 1980), POPG (Fleming et al., 1983), and PI (Fagan & Keough, 1988) under initial "surface dilute" conditions. The effectiveness of SP-B and SP-C in improving the respreadability of DPPC, as judged by the collapse plateau length ratios, was compared with those of dipalmitoylphosphatidylglycerol (DPPG) and cholesterol, measured under similar experimental conditions (Table 1). The effect of 30 wt % DPPG was comparable to those of 0.5 wt % SP-B or 2.5 wt % SP-C. Cholesterol, on the contrary, seemed to decrease the respreadability of DPPC at "surface dilute" concentrations. Snik et al. (1968) found that, under similar initial conditions, 10–20 mol % cholesterol did not influence the stable collapse phase of DPPC. At initial "surface excess" concentrations, cholesterol showed no effect on respreading of DPPC (Table 2), similar to the findings of other authors (Notter et al., 1980b). Films of pure DPPG (Table 1) and pure cholesterol (Snik et al., 1978; Notter et al., 1980b) showed enhanced respreading after collapse in comparison to DPPC films alone. Mixed films of DPPG/DPPC and cholesterol/DPPC studied here displayed a single collapse at surface pressures of about 72 mN m^{-1} (results not shown). Therefore, both DPPG and cholesterol were likely incorporated into the collapse structures of DPPC, similar to the case of hydrophobic protein/DPPC films. Inspection of the collapse plateau length ratios in Table 1 showed that incorporation of a second component into the collapse phase of DPPC did not necessarily increase its respreadability. It is worth noting that both DPPG and the hydrophobic proteins perturbed the molecular packing in the films of DPPC up to $\pi \approx 65$ mN m^{-1} and this led to expansion in the mean molecular areas in the mixed films (Taneva & Keough, 1994a,b). In agreement with the data in Table 1, one might speculate that such an impact of a second component on the monolayers of DPPC would result in destabilization and hence increase respreading of the collapses structures of DPPC. The mean molecular area plots for cholesterol/DPPC monolayers were additive at π > 20 mN m^{-1} (Shah & Schulman, 1968). In this case, the collapse plateau length ratios in Table 1 showed that the second component did not improve respreading of DPPC. These preliminary results suggest that a second component which

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

monolayer	concentration of non-DPPC component			initial spreading surface pressure[c] (mN m^{-1})	collapse plateau length ratios	
	wt %	mol %	X_r[b]		2/1	4/1
DPPC				47.0	0.34	0.15
SP-B/DPPC	0.5	0.02	0.03	47.8	0.45	0.28
SP-B/DPPC	1.0	0.04	0.06	46.8	0.51	0.29
SP-B/DPPC	2.5	0.11	0.15	45.2	0.54 (1.08)[d]	0.33 (0.89)
SP-B/DPPC	10.0	0.47	0.43	45.0	0.53	0.36
SP-C/DPPC	0.5	0.09	0.03	47.3	0.40	0.20
SP-C/DPPC	1.0	0.18	0.06	48.2	0.46 ± 0.01[e]	0.22 ± 0.02
SP-C/DPPC	2.5	0.45	0.14	45.0	0.53	0.33
SP-C/DPPC	5.0	0.92	0.25	46.9	0.67	0.55
SP-C/DPPC	10.0	1.92	0.41	45.5	0.70 ± 0.02	0.67 ± 0.03
DPPG/DPPC	10.0	9.8		46.5	0.43	0.28
DPPG/DPPC	30.0	29.7		44.9	0.57 ± 0.01	0.53 ± 0.01
DPPG				38.1	0.59	0.46
cholesterol/DPPC	8.0	14.2		48.0	0.37 ± 0.02	0.11 ± 0.02

[a] In each experiment, maximum surface pressure of 72 ± 1 mN m^{-1} was attained at the end of compression, and surface pressure was >0 mN m^{-1} at the end of expansion. [b] X_r is the fraction of amino acid residues of the protein in the mixed monolayers. [c] The initial spreading pressure corresponded to 0.33 nm^2 per molecule of DPPC at the start of compression. [d] The values in parentheses represent the collapse plateau length ratios determined from experiments where surface pressure was near zero at the end of expansion (see Figure 6a). [e] The values represent mean ± SD (n = 3).

decreases the molecular packing density of DPPC monolayer might be expected to impart better respreading of the collapse phase of the phospholipid.

In a second series of experiments, the films of pure DPPC and the binary lipid−protein films were formed under initial "surface excess" conditions. For the experiments here, at the start of dynamic compression, at the area A_{excess} (Figure 5a−d), each of the monolayers contained the same amount of DPPC, corresponding to 0.33 nm^2 per molecule of DPPC. This area was below the monolayer collapse area for DPPC, approximately 0.40 nm^2/molecule. At these surface concentrations in the films, constant surface pressures were obtained and they did not change upon spreading of additional aliquots as long as the surface area was not changed. The cycling time was 30 s, and the relative change in the monolayer area was 57%. Isotherms for the cyclic compression−expansion of films of pure DPPC starting in "surface excess" conditions are shown in Figure 5a. The shifts of the second and fourth compression curves toward lower areas were consistent with irreversible loss of material during monolayer collapse. When the films at the end of the fourth expansion, corresponding to the area A_{excess}, were additionally expanded to higher areas, the surface pressure reached zero. Therefore, the nonzero values of surface pressure at the end of other expansions were not an artifact due to changes in the contact angle of the Wilhelmy plate.

The lengths of the collapse plateaux were determined and their ratios were recorded in Table 2. The cycle 2/cycle 1 ratio for DPPC was comparable to those previously reported (Notter et al., 1980a; Turcotte et al., 1977). Comparison of the collapse plateau length ratios for DPPC in Tables 1 and 2 showed a greater degree of respreading of films formed at low initial concentrations than that found for films spread at initial "excess" concentrations. Data in other studies have shown a similar tendency for the films of DPPC at 23 °C (Notter et al., 1980a) and 35−37 °C (Notter et al., 1980b; Fleming et al., 1983) and also for the films of DPPC plus unsaturated PG (Notter et al., 1980a; Fleming et al., 1983). The reasons for this behavior are not understood, but it might indicate that the excess lipid deposited on initial spreading formed a different three-dimensional phase(s) than that formed on monolayer collapse with potentially different spreading rates.

The dynamic behavior of the DPPC films containing either SP-B or SP-C under "surface excess" conditions is shown in Figure 5b−d. The addition of the hydrophobic protein led to improved respreadability of DPPC which was readily seen in the isotherms, where the second and fourth cycles for the mixed films displayed greater hysteresis than did those for the films of pure DPPC (Figure 5a). The surface pressure at the end of expansion of the lipid−protein films, area A_{excess}, did not reach zero (Figure 5b−d), similar to the films of DPPC alone (Figure 5a). The collapse plateau length ratios for the protein−lipid films, summarized in Table 2, show that both SP-B and SP-C promoted the respreading of DPPC from collapse phase, similar to the effects seen under initial "surface dilute" conditions. The effect of SP-B on the respreadability of DPPC was essentially equivalent to that observed at "surface dilute" concentrations (Table 1). Thus, comparison of the plateau length ratios for the SP-B/DPPC films to those of DPPC alone showed that SP-B increased the respreading of DPPC about 1.5−2 times at both surface "dilute" and "excess" concentrations. The effect of 2.5 wt % of SP-C on the respreadability of DPPC was similar to that of the same concentration of SP-B. Further increase in the concentration of SP-B did not improve the respreadability of DPPC, as opposed to SP-C which did have greater effect at the high concentrations. Comparison of the collapse plateau ratios for SP-C/DPPC films containing 5 and 10 wt % SP-C to that for pure DPPC showed an enhancement of respreading of the lipid−protein films of about 2−4 times over that of the pure lipid (Table 2). At lower concentrations (≤2.5 wt %) the effects of SP-C on respreading of DPPC were similar at the two initial spreading conditions.

We do not know why SP-C, when present at higher concentrations (≥5 wt % or $X_r \geq 0.25$), facilitated the respreading of DPPC to a greater degree under initial "excess" conditions compared to the "dilute" ones. Comparison of the cyclic pressure−area curves for SP-C/DPPC films of composition $X_r = 0.41$, or 10 wt % protein, formed under the two initial conditions suggested that the collapse behavior of the protein in the binary films was possibly

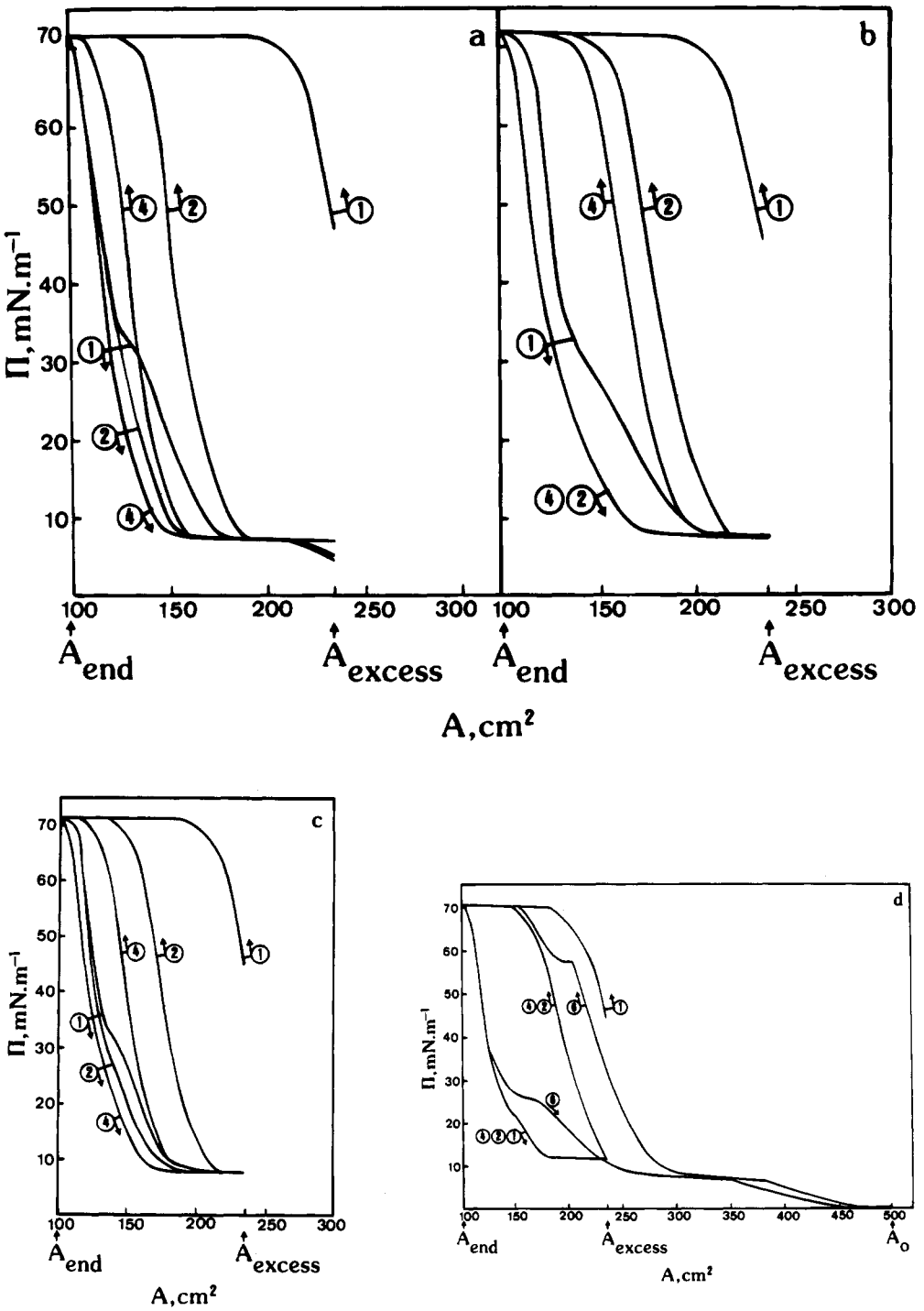

FIGURE 5: Dynamic compression–expansion behavior of "surface excess" films of DPPC (a); DPPC plus 2.5 wt % SP-B, or $X_r = 0.15$ (b); DPPC plus 2.5 wt % SP-C, or $X_r = 0.14$ (c); DPPC plus 10 wt % SP-C, or $X_r = 0.41$ (d). Cycles 1, 2, and 4 are shown. Initial spreading concentrations corresponded to 0.33 nm^2 per DPPC molecule. Relative change in the area was about 57% and cycling time was 30 seconds. Figure 5d also shows the sixth compression–expansion isotherm which was measured after the fifth expansion of the SP-C/DPPC films to zero surface pressure at the area A_0.

affected by the mode of formation of the initial film before the start of dynamic compression. Thus, the surface pressure–area compression curves for SP-C/DPPC films which were formed under "dilute" conditions (Figures 2c, 3d, and 4d) showed plateau regions at about 55 mN m^{-1}, consistent with exclusion of SP-C–DPPC complexes. The compression isotherms for the SP-C/DPPC films of identical initial composition, spread at initial "surface excess" concentrations (Figure 5d, curves 1, 2, and 4), showed no squeeze-out plateaux. Likewise, the first, second, and fourth expansion isotherms in Figure 5d did not exhibit inflections at about 30 mN m^{-1}, associated with respreading, compared to the well-pronounced ones in the expansion curves for the films of similar composition formed under "dilute" conditions (Figures 2c, 3d, and 4d). The expansion isotherms in Figure 5d suggested that at the end of expansion, at the area A_{excess}, the respreading of the collapse phase was not completed for either of the four cycles.

It is worth noting that the difference in the behavior of the films formed under different initial spreading conditions was not due to the various rates of compression used in the two sets of experiments, e.g., in the case of "surface excess"

films the rate of compression was 2.5 times higher than in "surface dilute" ones. This was verified in separate experiments where the films of SP-C/DPPC ($X_r = 0.41$) formed at initial "surface dilute" concentrations showed squeeze-out plateau on compression at $\pi \approx 55$ mN m^{-1} when a compression rate identical to that in the "surface excess" experiments was used (data not shown). Therefore, the initial spreading conditions appeared to affect the collapse behavior of the SP-C/DPPC films of higher protein concentrations, so that squeeze-out of SP-C was not apparent from the pressure−area curves for the "surface excess" films in contrast to the "surface dilute" films. This change in the collapse properties of SP-C possibly caused the enhanced effect of SP-C on the post-collapse respreading of DPPC under "surface excess" concentrations over that of "surface dilute" ones. The mechanistic reason for these observations is unknown, and we can only speculate that the formation of the "surface excess" films possibly affected either the orientation of SP-C in the lipid−protein films or the distribution of the protein between the monolayer phase and the collapse phase formed during the spreading process before the start of dynamic compression.

The following experiment, the results of which are seen in Figure 5d, supported the idea that the surface pressure at start of compression affected the lipid−protein interactions in the films. At the end of the fifth expansion of the "excess" SP-C/DPPC films, at the area A_{excess} and $\pi \approx 12$ mN m^{-1}, the films were further expanded to the larger area A_0, so that zero surface pressure was attained at the end of expansion. Then the films were compressed to the minimum area A_{end} and reexpanded to the area A_0 (cycle 6 in Figure 5d). The sixth compression isotherm, as opposed to the preceding ones, exhibited a squeeze-out plateau at about 57 mN m^{-1}. The sixth expansion isotherm, showed an inflection at about 27 mN m^{-1} associated with respreading. These findings suggested that the fifth expansion of the film to zero surface pressure led to more complete respreading of the collapse phase and possibly to reorientation and realignment of the protein (and lipid) molecules in the monolayer, so that upon the subsequent compression a squeeze-out plateau, typical for films formed under "surface dilute" conditions, was seen. The shift of the sixth compression curve toward higher areas, when compared to the second and fourth ones, also implied that the expansion of the films to low surface pressure improved the respreading of the postcollapse phases.

This finding suggested a possible explanation for the considerable difference in the respreading properties of the films of pure DPPC and those of DPPC plus hydrophobic protein under "surface dilute" and "surface excess" conditions. Comparison of the data in Tables 1 and 2 showed the enhanced respreadability of the "dilute" films over their "excess" counterparts. Comparison of the compression and expansion isotherms for the two series of initial spreading conditions showed that, on the second and the higher expansions, the surface pressure reached zero in the "surface dilute" films (Figure 4a−d). For the "surface excess" films, the surface pressure at the end of the expansion never reached a value lower than the pressure corresponding to the phase transition between liquid-condensed and liquid-expanded states (Figure 5a−d).

To check whether the respreading of the collapse phases formed during compression of the "surface excess" films would be improved by exposure of the postcollapse phases

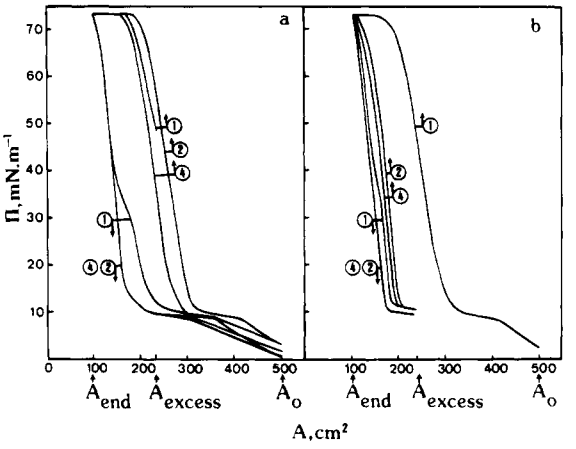

FIGURE 6: (a) Dynamic compression−expansion cycles of "surface excess" films of DPPC plus 2.5 wt % SP-B, or $X_r = 0.15$, where surface pressure was near zero at the end of expansion. Relative change in the area was 80% and cycling time was 90 s. (b) Compression−expansion cycles for "surface dilute" films of SP-B/DPPC, $X_r = 0.15$, where at the end of expansion surface pressure was above the pressure associated with the phase transition from liquid-condensed to liquid-expanded state.

to low (near zero) surface pressure, the following experiment was performed (Figure 6a). Films of DPPC plus 2.5 wt % SP-B were formed at the area A_{excess}, at initial "surface excess" concentrations, identical to those in Figure 5b. At the end of the first cycle, they were expanded to the area A_0 where low surface pressure corresponding to liquid-expanded state of the films was reached, and then three compression−expansion cycles were performed between the areas A_0 and A_{end}. The collapse plateau length ratios for the films in Figure 6a, shown in parentheses in Table 2, demonstrated that expansion of the "surface excess" films to surface pressures below the pressure of the liquid-condensed to liquid-expanded transition increased the respreading of the postcollapse phases and yielded values for the collapse plateau length ratios comparable to those for the films of similar compositions formed under "surface dilute" conditions (compare Table 1). From these results it appeared that possibly the molecular aggregates formed during collapse of the films could not disassociate completely when the lateral pressure at the end of expansion was higher than, or equal to, the pressure corresponding to the liquid-expanded to liquid-condensed phase transition (Figure 5a−d). Complete dissociation of the molecular aggregates, i.e., release of molecules into the monolayer and, possibly, reorientation of the molecules at the low pressures, occurred when the films were expanded to surface pressures corresponding to their liquid-expanded state (Figures 5d and 6a). These processes may account for the improved respreadability of the "surface excess" films when they were expanded to surface pressures near zero after compression past collapse.

In another experiment, films of DPPC plus 2.5 wt % SP-B were formed at area A_0, at initial "surface dilute" concentrations similar to those in Figure 4b. They were compressed beyond collapse and then were cycled between the areas A_{excess} and A_{end} (Figure 6b). In this case, at the end of expansion, surface pressure did not reach values below the pressure associated with transition from liquid-condensed to liquid-expanded state. The collapse plateau length ratios for the SP-B/DPPC films in Figure 6b, shown in parentheses in Table 1, revealed that under these experimental conditions the "surface dilute" films of SP-B/DPPC exhibited lower

respreadability compared to films of identical initial composition, formed under similar initial conditions, which were expanded to zero surface pressure at the end of expansion (Figure 4b). The results in Figure 6a,b suggested that expansion of the films to liquid-expanded state at the end of the preceding cycle was a factor that determined the ability of both "surface dilute" and "surface excess" films to respread after compression past collapse.

CONCLUSIONS

The monolayers of the hydrophobic pulmonary surfactant proteins, SP-B and SP-C, showed reversible collapse during dynamic cyclic compression−expansion. These proteins were reversibly excluded and reinserted in their binary monolayers with DPPC when the films were compressed just bellow the collapse pressure of the phospholipid. When the lipid−protein films were compressed past the collapse of DPPC, SP-B and SP-C imparted better respreadability to the excluded phases, without affecting the ability of DPPC to achieve maximal dynamic surface pressure of about 72 mN m^{-1}. We do not know the exact mechanism(s) by which SP-B and SP-C acted to improve the respreading of DPPC. Perhaps, at low protein concentrations, e.g., 2.5 wt %, the proteins which were incorporated into collapse structures of DPPC destabilized them and thus increased their respreadability. At high protein concentrations, e.g., 10 wt %, where separate collapse (exclusion) of the proteins was seen in addition to the above mechanism, some phospholipid was possibly incorporated into the protein collapse structures which were reversibly excluded at the low pressures. In this case, that part of DPPC molecules, associated with the protein, was possibly protected from irreversible collapse at 72 mN m^{-1} upon compression and reinserted into the monolayer together with the protein upon subsequent expansion. This particular mechanism would operate mostly in SP-C/DPPC films, since SP-C removed a larger amount of phospholipid than did SP-B from SP-B/DPPC films.

In films formed at initial "surface dilute" concentrations, SP-B enhanced the post-collapse respreading of DPPC better than did SP-C. In films formed at initial "surface excess" concentrations when protein concentrations were ≥5 wt %, SP-C showed greater ability to improve respreading than did SP-B. The conditions of performing the dynamic cyclic pressure−area measurements, e.g., the spreading pressure at the start of compression and the pressure at the end of expansion, affected the respreading ability of the films of DPPC alone and those of DPPC plus hydrophobic protein. The ability of the "surface excess" films to respread from excluded phases was considerably improved when low (zero) surface pressure was reached at the end of expansion of the films which had been compressed beyond the point of collapse. This procedure yielded high collapse plateau length ratios for the films, comparable to those found for their counterparts formed under "surface dilute" conditions. This would imply that a large sigh could improve respreading of any collapse phase formed *in vivo*.

In conclusion, the results in the present paper suggest that, if postcollapse respreading is a route for replenishment of lipid at the air−water interface in lung, SP-B and SP-C would be effective agents in promoting this activity. *In vitro* studies have shown that the two pulmonary surfactant proteins impart surface properties to DPPC, the primary phospholipid component of lung surfactant, which are essential for the function of lung surfactant *in vivo*. In a simplified chain of interfacial events during the breathing cycle in lung alveoli, both SP-B and SP-C might enhance the rate of adsorption of phospholipids from three-dimensional associations in the subphase into the monolayer (Suzuki, 1982; Takahashi & Fujiwara, 1986; Yu & Possmayer, 1986). The results in this and earlier papers (Taneva & Keough, 1994a,b,c) suggested that the hydrophobic proteins would not interfere with the ability of DPPC to sustain high surface pressures in alveolar monolayers attained during expiration at maximum compression. Upon inspiration, SP-B and SP-C may improve the respreading of DPPC from possible collapse structures formed at the interface. Thus, it appears that SP-B and SP-C may have multiple functions in the events occurring at the alveolar−air interface. It seems legitimate to expect that an effective additive to DPPC for surfactant replacement therapy would have to reflect the complex impact of the specific hydrophobic pulmonary surfactant proteins on the surface properties of DPPC.

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