Dynamic surface activity of films of lung surfactant phospholipids, hydrophobic proteins, and neutral lipids

Zhengdong Wang,* Stephen B. Hall, and Robert H. Notter[1,*,†]**

Department of Pediatrics* and Environmental Medicine,† University of Rochester, 601 Elmwood Avenue, Rochester, NY 14642, and Department of Internal Medicine,** Oregon Health Sciences University, Portland, OR 97201

Abstract Surface pressure-area (π-A) isotherms during dynamic cycling were measured for films of dipalmitoyl phosphatidylcholine (DPPC) and column-separated fractions of calf lung surfactant extract (CLSE). Emphasis was on defining the relative importance of lung surfactant phospholipids (PPL), neutral lipids (N), and hydrophobic proteins (SP) in facilitating dynamic respreading and surface tension lowering within the interfacial film itself. Solvent-spread films in a Wilhelmy balance were studied at 23° and 37°C over a range of cycling rates for initial concentrations giving both monomolecular and surface-excess films. A striking finding was that PPL films containing the complete mix of surfactant phospholipids had greatly improved dynamic respreading compared to DPPC, particularly in surface excess films (30 and 15 Å^2/molecule). Hydrophobic SP gave an additional increase in dynamic respreading in SP&PL compared to PPL films for initial concentrations of 60, 30, and 15 Å^2/molecule. Neutral lipids also improved respreading slightly in N&PL versus PPL films, but maximum surface pressures in N&PL films at 37°C were consistently the lowest of any surfactant subfraction. Spread films of SP&PL at 60 and 30 Å^2/molecule had lower maximum pressures than PPL, but maximum pressures were slightly larger for SP&PL films at high initial concentration (15 Å^2/molecule). Supplementary oscillating bubble studies involving both adsorption and film dynamics at rapid cycling rate (20 cycles/min) showed that dispersions of CLSE and SP&PL lowered surface tension to <1 mN/m, while PPL and N&PL had elevated minimums of 21 mN/m. These results show that secondary surfactant phospholipids in addition to DPPC are important in the film behavior of pulmonary surfactant, giving improved respreading and overall π-A isotherms very different from disaturated phospholipids. Hydrophobic SP also increase respreading in the interfacial film, in addition to their known action in increasing surfactant adsorption. SP may also improve film stability at high interfacial concentrations of phospholipid, although they were destabilizing in more dilute films. Neutral lipids contributed minor increases in surfactant respreading, but were consistently detrimental to surface tension lowering.—**Wang, Z., S. B. Hall, and R. H. Notter.** Dynamic surface activity of films of lung surfactant phospholipids, hydrophobic proteins, and neutral lipids. *J. Lipid Res.* 1995. **36:** 1283–1293.

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Chromatographic methodology now makes it feasible to separate hydrophobic lung surfactant extracts into subfractions from which specific components are selectively excluded (1). Studies of the surface-active behaviors of these subfractions then allow the roles and importance of individual molecular constituents in lung surfactant activity to be more clearly delineated. Pulmonary surfactant is a complex mixture of lipids and proteins (2–6). In order to stabilize alveoli and reduce the work of breathing, its molecular components must interact to generate a set of surface-active behaviors (2–4). These include rapid adsorption to the air–water interface after secretion into the alveolar hypophase, and the formation of a surface film able to lower surface tension and respread effectively during successive cycles of dynamic compression and expansion (3, 4). The present study examines the actions and relative importance of different hydrophobic constituents of lung surfactant within the interfacial film itself during continuous dynamic cycling.

Mammalian lung surfactant is complex in composition. In addition to the major phospholipid dipalmitoyl phosphatidylcholine (DPPC) and surfactant proteins (SP)-A, B, and C, it contains a variety of saturated and unsaturated phospholipids, plus neutral lipids including cholesterol (3, 6, 7). DPPC accounts for only part of the overall surface activity of lung surfactant. Interfacial films of DPPC are known to lower surface tension to extraordinarily low values <1 mN/m during dynamic compression (8–10), and this disaturated phospholipid is felt to be the primary surface tension-lowering component of functional surfactant. However, DPPC does not adsorb

Abbreviations: DPPC, dipalmitoyl phosphatidylcholine; CLSE, calf lung surfactant extract; PPL, purified phospholipids; N&PL, neutral lipids + phospholipids; SP, hydrophobic proteins; SP&PL, hydrophobic proteins + phospholipids.

[1]To whom correspondence should be addressed.

readily to the air–water interface (11, 12), and this is facilitated in lung surfactant by the surfactant proteins (SP)-A, -B, and -C (13–16). DPPC also exhibits very low dynamic respreading in surface films compressed in the collapse regime (8, 9). Molecules of DPPC squeezed-out of the interface at high surface pressure (low surface tension) during compression are not able to re-enter the film during subsequent expansion, and are no longer available to participate in surface tension lowering (5, 8, 9). The relative importance of surfactant proteins, secondary phospholipids, and neutral lipids in facilitating the respreading of DPPC is not well defined.

This study examines in detail the surface tension lowering and respreading behavior of films of calf lung surfactant extract (CLSE), containing all the hydrophobic constituents of whole surfactant, and of selected fractions of CLSE prepared by column chromatography (1): purified phospholipids alone (PPL), neutral lipids plus phospholipids (N&PL), and surfactant proteins and phospholipids (SP&PL). Unsaturated zwitterionic and anionic phospholipids have been shown to influence DPPC film behaviors including respreading (8, 17–19), but the surface active properties of interfacial films of PPL, N&PL, and SP&PL have not been studied directly. The major focus of our experiments is on Wilhelmy balance studies isolating effects within spread interfacial films of CLSE, PPL, N&PL, and SP&PL, with adsorption-related phenomena minimized. Additional oscillating bubble measurements define the activity of surfactant dispersions pulsated at rapid rate, under conditions involving both adsorption and film dynamics. The results show that secondary lung surfactant phospholipids, in addition to DPPC and apoproteins, have an important influence on film properties, particularly dynamic respreading. These mixed phospholipids may be important not only in endogenous lung surfactant activity, but also for the future development of more optimal exogenous surfactants.

MATERIALS AND METHODS

Surfactant materials

Surfactants used were 1,2-dipalmitoyl-*sn*-3-phosphocholine (DPPC), calf lung surfactant extract (CLSE), and chromatographically purified fractions of CLSE. DPPC was purchased as >99% pure from Avanti Polar Lipids, Inc. (Alabaster, AL), with purity verified by a single spot on thin-layer chromatography (TLC) on silica gel G with a solvent system of chloroform–methanol–2-propanol–water–triethylamine 30:9:25:7:25 (by volume) (20). Lung surfactant was obtained by saline lavage of intact lungs from calves (Conti Packing Co., Henrietta, NY), as described previously (12, 21, 22). Lavage was immediately centrifuged at low speed (250 *g* for 10 min) to remove cells and debris, and at higher speed (12,500 *g* for 30 min) to pellet surface-active aggregates. Pelleted whole surfactant was resuspended and extracted into chloroform–methanol (23) to give CLSE. The same parent CLSE batch in **Table 1,** having an overall composition equivalent to similar extracts previously described from our laboratory (1, 3, 21, 22), was used for isolation of all the lung surfactant hydrophobic subfractions of the present study.

Fractions of CLSE were isolated by gel permeation column chromatography (1). A single pass through a 1 × 50 cm column of LH-20 (Pharmacia-LKB Biotechnology, Piscataway, NJ) in chloroform–methanol–0.1 N HCl 47.5:47.5:5 separated hydrophobic surfactant proteins and phospholipids (SP&PL) from neutral lipids. Two passes were required to prepare fractions of purified phospholipids alone (PPL) or neutral and phospholipids (N&PL) containing minimal SP. Final N&PL and PPL subfractions contained protein below the limits of detection by amido black staining of trichloroacetic acid-precipitated material (24) (<0.06% by weight protein relative to phospholipid based on assay limits). We have shown previously that the process of separation does not alter these different fractions biophysically, and reconstituted CLSE (rCLSE) made from them has surface active behavior indistinquishable from parent CLSE (1). Lipid content and composition in separated fractions were defined by TLC (20), cholesterol assay (25), and phosphate assay (26). PPL and N&PL had a phospholipid distribution equivalent to CLSE (Table 1), and neutral lipids in N&PL were at the same ratio of cholesterol to phospholipid found in CLSE (85.1 ± 3.8 and 83.6 ± 3.4 nmol/μmol, respectively) (1). Extraction of chromatographic samples to remove acid limited recovery of protein in SP&PL, and final SP&PL subfractions were supplemented with SP purified separately to restore the protein-to-phospholipid ratio to the original level of 1.3% by weight in CLSE (1).

TABLE 1. Biochemical composition of calf lung surfactant extract (CLSE)

	% Total (by wt)
A. Class analysis	
Phospholipids	94.2
Neutral lipids	4.5
Proteins (SP-B, C)	1.3
B. Phospholipid composition	
Phosphatidylcholine	83.5
Phosphatidylglycerol	4.5
Phosphatidylinositol	4.2
Phosphatidylethanolamine	3.2
Sphingomyelin	2.0
Residue	2.6

Neutral lipids, primarily composed of cholesterol along with traces of cholesterol ester (3), were determined by cholesterol assay (25). Data are means from four independent determinations.

Wilhelmy balance measurements

Surface pressure-area (π-A) isotherms of solvent-spread interfacial films were measured with a modified Wilhelmy surface balance incorporating a continuous ribbon barrier to minimize leakage as described by Tabak and Notter (27). Highly pure distilled and deionized water (Milli-Q UV Plus system, Millipore Corp, Bedford, MA) was used in all balance cleaning procedures and for subphase formation (150 mM NaCl, 10 mM HEPES, 1.5 mM $CaCl_2$, pH 7.0). In a typical experiment, surfactants dissolved in hexane-ethanol 9:1 (v/v) (HPLC grade, VWR Scientific Co., Philadelphia, PA) were spread dropwise from a syringe at the air-water interface to a standard interfacial concentration (150, 120, 60, 30, or 15 Å^2/phospholipid molecule). Dynamic cycling commenced after a 10-min pause for solvent evaporation, with surface pressure (surface tension lowering) monitored continuously from the force on a sand-blasted platinum slide dipped into the ribbon-enclosed surface. Film leakage was monitored by a second slide outside the ribbon barrier, and was absent in all π-A results reported. Up to seven successive cycles of compression/expansion at the interface were measured between maximum and minimum areas of 448 and 103 cm^2 (compression ratio 4.35:1), at speeds of 10, 5, or 1.5 min per complete cycle. These area conditions led to extensive compression in the collapse regime for films spread to high initial concentration (60, 30, 15 Å^2/molecule), resulting in maximal surface tension lowering and emphasizing film respreading behavior. Experimental temperature was maintained at 23 ± 1 or 37 ± 0.5°C, and humidity was held near the fully saturated value by several open dishes of water and dampened blotting paper in the environmental chamber.

Dynamic respreading was characterized from π-A isotherm areas, modifying the previously defined collapse plateau ratio criteria of Notter and co-workers (8, 9, 19) to describe more accurately the behavior of multicomponent surfactant films with varying slopes during compression (**Fig. 1**). As illustrated, the π-A isotherm area between the first and second compression curves was used to approximate surfactant material lost from the interface during cycle 1 that did not respread and participate in surface tension lowering during compression 2. Similarly, the area between compressions 1 and 7 reflected interfacial material lost during the first six cycles of the film. Isotherm areas were calculated in arbitrary but consistent units to allow respreading comparisons between films of different surfactants spread to the same initial concentration and compressed to the same end-point. A calculated isotherm area of zero between compression curves 2/1 and 7/1 indicated complete respreading, and respreading decreased as area increased. The poor respreading of pure DPPC films gave an effective upper limit on the isotherm areas of the other surfactant films.

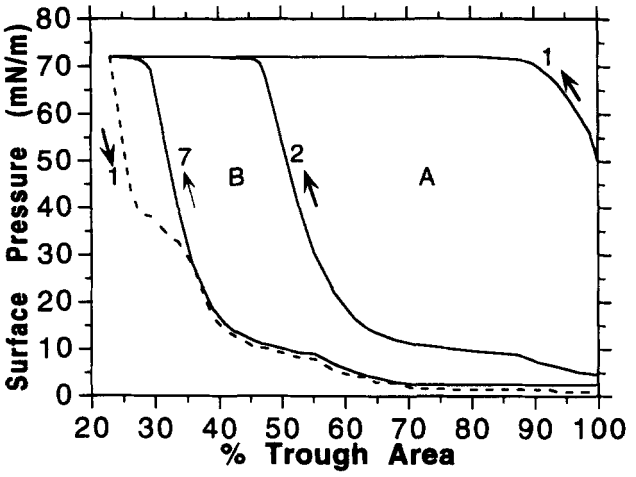

Fig. 1. Isotherm area calculation of respreading. The dynamic surface pressure-area (π-A) isotherm shown has an area "A" between compression curves 1 and 2 (solid lines), and an area "A + B" between compressions 1 and 7. As described in the text, these areas give a measure of film material lost from the interface on first compression (area between curves 2 and 1) or during the first six compressions (area between curves 7 and 1), i.e., film material that did not respread by compression 2 or 7. Comparison of isotherm areas (in consistent but arbitrary units) for different surfactant films spread to the same initial area and compressed equivalently allows relative respreading facility to be assessed. The isotherm shown is representative of DPPC (15 Å^2/molecule, 23°C), with large areas A,B indicating poor respreading. A film that respreads completely would have A,B = 0.

Oscillating bubble methods

A pulsating bubble surfactometer (Electronetics, Inc., Amherst, NY) based on the design of Enhorning (28) was used to define the overall surface activity of dispersions of CLSE and its subfractions at rapid cycling rate (20 cycles/min). Surfactant samples were dispersed by probe sonication on ice in 10 mM HEPES, pH 7.0, 0.15 M NaCl, 1.5 mM $CaCl_2$ at a concentration of 0.75 μmol phospholipid/ml, and bubble studies were performed at 37°C.

RESULTS

Dynamic respreading for DPPC, CLSE, PPL, SP&PL, and N&PL films is quantitated in terms of π-A isotherm areas in **Table 2** for films compressed at constant rate (1.5 min/complete cycle) from initial concentrations of 60, 30, and 15 Å^2/molecule at 37°C. For all initial concentrations, calculated isotherm areas between compressions 2 and 1 were decreased substantially for PPL films compared to DPPC, indicating that PPL had greatly improved dynamic respreading (Table 2). Respreading for PPL films was much closer to CLSE than to DPPC, particularly for films spread initially at high surface concentration (30 and 15 Å^2/phospholipid molecule, Table 2). Films of PPL and CLSE both had very small area differences between compressions 2 and 1, with values de-

TABLE 2. Respreading of lung surfactant subfraction films at a fixed cycling rate of 1.5 min/cycle at 37°C

Films	Initial Conc. (Å²/m)	Respreading Based on Isotherm Areas between Compression Cycles	
		2 & 1	7 & 1
DPPC	60	33.5 ± 0.3	54.5 ± 0.3
PPL	60	21.1 ± 0.3	48.3 ± 0.7
N&PL	60	20.7 ± 0.3	47.2 ± 0.3
SP&PL	60	9.3 ± 0.5	27.9 ± 0.4
CLSE	60	2.3 ± 0.3	20.2 ± 0.7
DPPC	30	29.6 ± 0.1	48.0 ± 0.2
PPL	30	0.8 ± 0.1	38.5 ± 0.5
N&PL	30	0.6 ± 0.1	29.3 ± 0.4
SP&PL	30	0.7 ± 0.0	27.5 ± 0.4
CLSE	30	0.3 ± 0.0	16.0 ± 0.4
DPPC	15	29.6 ± 0.2	47.4 ± 0.2
PPL	15	0.6 ± 0.0	27.4 ± 0.5
N&PL	15	0.1 ± 0.0	17.6 ± 0.3
SP&PL	15	0.2 ± 0.0	11.0 ± 0.7
CLSE	15	0.2 ± 0.0	5.2 ± 0.1

Solvent-spread films were compressed in a Wilhelmy balance at 1.5 min/complete cycle at 37°C. Data are mean ± SEM for n ≥ 4. The isotherm area calculation (arbitrary units) for respreading is diagrammed in Fig. 1.

creased 40-fold or more compared to DPPC for initial spreading to 30 and 15 Å²/molecule at 37°C. Area differences between compressions 7 and 1 for PPL films were increased and elevated compared to CLSE, but still remained significantly lower than for DPPC (Table 2).

The respreading calculations in Table 2 reflected major differences in the shapes of the entire π-A isotherms of DPPC and PPL. Representative π-A isotherms for films of DPPC, CLSE, PPL, N&PL, and SP&PL are shown in **Fig. 2** for initial spreading to 15 Å²/molecule (first complete cycle and second and seventh compressions). At this surface-excess concentration, and also at 60 and 30 Å²/molecule (not shown), DPPC films reached higher π values earlier in first compression than CLSE and its fractions, but surface pressures during expansion were significantly lower for DPPC. Subsequent compressions for DPPC films gave high surface pressures only very near end-compression, indicating very poor respreading on continuous cycling into the collapse regime in agreement with previous work (8, 9, 11). Films of PPL had significantly better respreading than DPPC, with differences becoming more apparent as initial film concentration increased (Table 2).

SP&PL films exhibited a further increase in respreading compared to PPL (Table 2, Fig. 2), indicating that hydrophobic SP gave an additional enhancement beyond the effects of the secondary phospholipids in PPL. The beneficial effects of hydrophobic SP on respreading were most apparent during prolonged cycling, and SP&PL films consistently had smaller isotherm areas between compressions 7 and 1 compared to PPL at 60, 30, and 15 Å²/molecule (Table 2, Fig. 2). Isotherm areas between compressions 2 and 1 were more similar for SP&PL and PPL films, particularly for initial spreading to high surface concentration (30, 15 Å²/molecule). Films of N&PL also showed slightly better respreading than PPL (Table 2), but maximum surface pressures were consistently lower in films containing neutral lipids (Fig. 2).

A similar pattern of improved respreading in PPL versus DPPC films, with a further increase in SP&PL films, was also found at other cycling rates and initial concentrations, and at room as well as body temperature. **Table 3** shows calculated π-A isotherm areas for films of CLSE and its subfractions examined at slower cycling rates of 10 min/cycle (60 Å²/phospholipid molecule) and 5 min/cycle (15 Å²/molecule) at both room and body temperatures. PPL films were again found to have consistently improved cycle 2/1 and 7/1 respreading over DPPC, with the difference more pronounced at high initial film concentration (Table 3). Respreading was also improved slightly by neutral lipids in N&PL, and more impressively by hydrophobic apoproteins in SP&PL films (particularly in terms of 7/1 respreading at 37°C in 15 Å²/molecule films, Table 3).

In addition to respreading, another important parameter for lung surfactant films is the maximum surface pressure (minimum surface tension) reached during cycling. For the films studied, surface pressure at fixed molecular area increased with cycling rate, with the magnitude of increase dependent on temperature, initial concentration, and film composition. **Table 4** shows the effects of cycling rate and temperature on the surface pressure at 80, 60, and 40 Å²/molecule, and at end-compression, for films of CLSE and its fractions spread initially to dilute initial concentration (150 Å²/molecule at 37°C or 120 Å²/molecule at 23°C). At room temperature, increasing the cycling rate from 10 min/cycle to 1.5 min/cycle gave an increase in surface pressure at fixed area for all films studied (Table 4). Increases in π with cycling rate were most pronounced in the high surface pressure regime near or after monolayer collapse, with smaller changes in π at large area/molecule. At body temperature, films were expanded, with isotherms shifted to higher area/molecule. For fixed cycling rate, films of DPPC and CLSE and its fractions had higher surface pressures at large area/molecule at 37°C compared to 23°C. However, π values within the collapse regime (including maximum π) were decreased at 37°C for films of CLSE and its fractions spread to dilute initial concentration (Table 4).

The maximum surface pressures reached during continuous cycling of more concentrated films of PPL, SP&PL, CLSE, and N&PL spread to 60, 30, and 15 Å²/molecule at 37°C are given in **Fig. 3** as a function of cycle number. At 23°C, maximum π values for films of PPL,

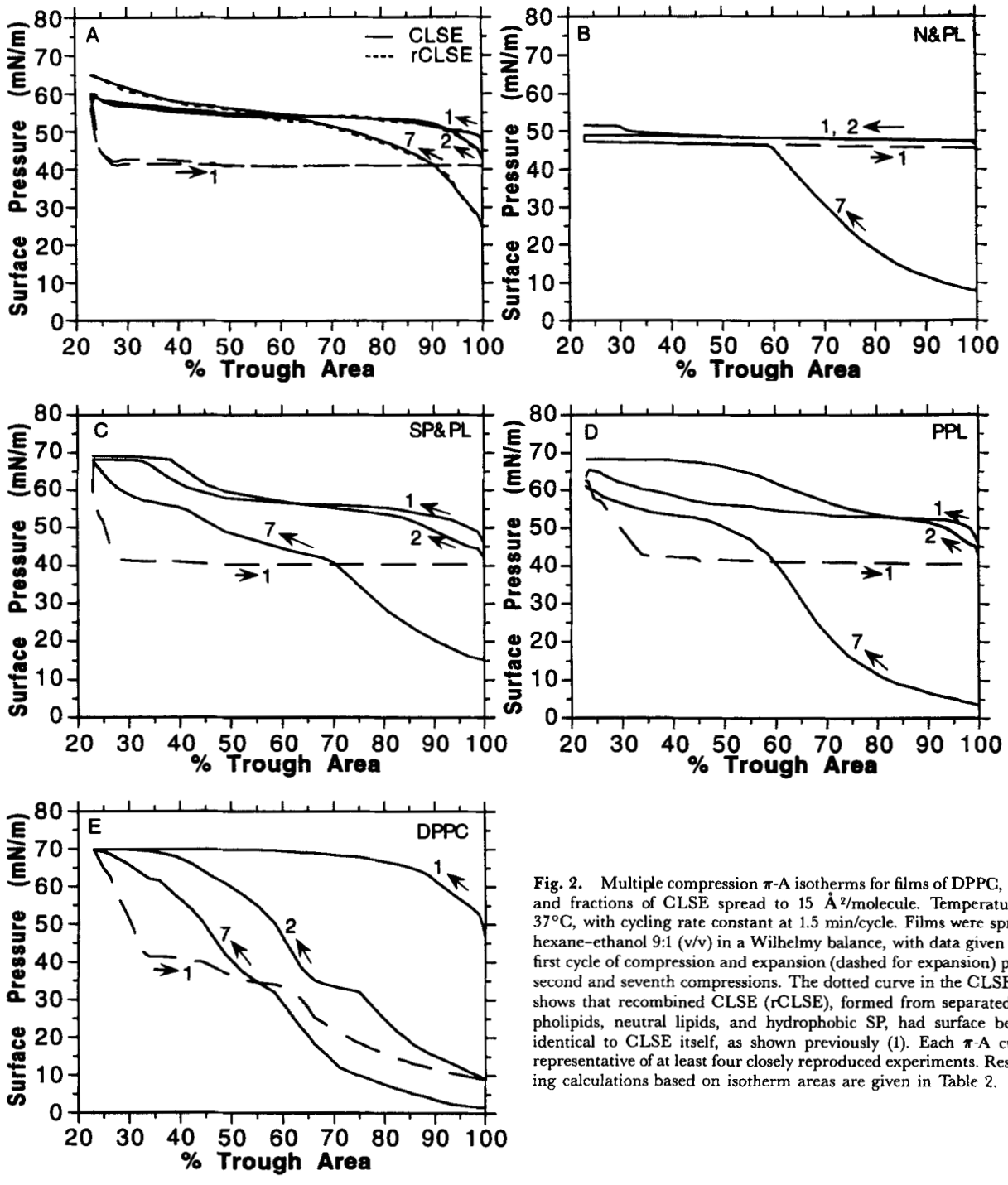

Fig. 2. Multiple compression π-A isotherms for films of DPPC, CLSE, and fractions of CLSE spread to 15 Å²/molecule. Temperature was 37°C, with cycling rate constant at 1.5 min/cycle. Films were spread in hexane–ethanol 9:1 (v/v) in a Wilhelmy balance, with data given for the first cycle of compression and expansion (dashed for expansion) plus the second and seventh compressions. The dotted curve in the CLSE panel shows that recombined CLSE (rCLSE), formed from separated phospholipids, neutral lipids, and hydrophobic SP, had surface behavior identical to CLSE itself, as shown previously (1). Each π-A curve is representative of at least four closely reproduced experiments. Respreading calculations based on isotherm areas are given in Table 2.

SP&PL, and CLSE decreased slightly on successive cycles, but were generally near to the high maximal pressure generated by DPPC (71.8 mN/m at 23°C, not shown on Fig. 3). N&PL films also generated substantial maximum pressures at 23°C, although they were less than for other subfractions particularly in later cycles (Fig. 3D). Maximum surface pressures decreased at body temperature compared to room temperature in all films of CLSE and its fractions (Fig. 3A–D). This effect was moderated by increased cycling rate and film concentration, however, for PPL, SP&PL, and CLSE. Maximum π values in these films increased as cycling rate increased from 10 to 1.5 min/cycle (60 Å²/molecule, Fig. 3) or from 5 to 1.5 min/cycle (15 Å²/molecule, Fig. 3). In films spread to

TABLE 3. Respreading of lung surfactant subfraction films at additional cycling rates at room and body temperature

Films	Initial Conc. (Å²/m)	T (°C)	Respreading Based on Isotherm Areas between Compression Cycles 2 & 1	7 & 1
DPPC	60	23	55.8 ± 0.3	64.4 ± 0.2
PPL	60	23	38.4 ± 0.4	53.0 ± 0.7
N&PL	60	23	35.8 ± 0.4	51.0 ± 0.3
SP&PL	60	23	18.4 ± 0.1	31.5 ± 0.1
CLSE	60	23	7.0 ± 0.2	24.6 ± 0.5
DPPC	60	37	28.8 ± 0.3	53.7 ± 0.5
PPL	60	37	21.2 ± 0.5	47.7 ± 0.5
N&PL	60	37	19.5 ± 0.3	43.8 ± 0.2
SP&PL	60	37	8.6 ± 0.5	23.5 ± 0.7
CLSE	60	37	3.7 ± 0.2	16.3 ± 0.4
DPPC	15	23	47.1 ± 0.2	70.2 ± 0.4
PPL	15	23	9.5 ± 0.7	49.0 ± 0.5
N&PL	15	23	4.6 ± 0.2	45.0 ± 0.1
SP&PL	15	23	3.4 ± 0.1	29.7 ± 1.0
CLSE	15	23	2.4 ± 0.1	30.0 ± 0.8
DPPC	15	37	28.3 ± 0.3	48.0 ± 0.3
PPL	15	37	0.2 ± 0.0	28.9 ± 0.3
N&PL	15	37	0.1 ± 0.0	15.5 ± 0.2
SP&PL	15	37	0.2 ± 0.0	7.7 ± 0.2
CLSE	15	37	0.2 ± 0.0	2.9 ± 0.2

Cycling rate was 10 min/complete cycle for 60 Å²/phospholipid molecule films, and 5 min/complete cycle for 15 Å²/molecule films. Other details as in the legend to Table 2.

15 Å²/molecule and compressed at 1.5 min/cycle at 37°C, DPPC reached the highest maximum surface pressure during cycling (69.9 ± 0.1 mNm, not shown), followed by SP&PL (69.1 ± 0.1 mN/m), PPL (68.4 ± 0.1 mN/m), CLSE (68.4 ± 0.3 mN/m), and N&PL (54.1 ± 0.3 mN/m). Films of N&PL consistently had the lowest maximum surface pressures of any of the surfactant subfractions studied, regardless of temperature, compression rate, initial concentration, or cycle number (Fig. 3D).

Effects of SP on maximum π were dependent on initial concentration and cycling rate, and were most beneficial in surface-excess films. For initial spreading to low concentration, SP&PL films had lower maximum pressures than PPL films during first compression at slow rate (end-compression in Table 4, 10 min/cycle, 23 and 37°C). Maximum pressures were also lower in 60 Å²/molecule films of SP&PL versus PPL compressed at more rapid rate (1.5 min/cycle, Fig. 3A, B). In additional studies, varying amounts of SP were added to PPL and DPPC prior to spreading to 60 Å²/molecule (**Table 5,** 1.5 min/cycle, 37°C). Addition of only 0.75% hydrophobic SP to PPL caused the maximum pressure of the mixed film to drop markedly, and further increases in the amount of SP from 1.3% to 5.2% gave no additional decrease (Table 5). SP had a lesser effect in films with DPPC at 60 Å²/molecule, giving smaller reductions in maximum π that were delayed to later cycles (5,7 in Table 5). As initial concentration increased to 15 Å²/molecule, SP raised maximum π slightly in SP&PL versus PPL films during the majority of compressions (Fig. 3A, B). Maximum surface pressure was increased in surface-excess SP&PL versus PPL films during the first and second compressions, and was maintained at a high level through more subsequent cycles (15 Å²/molecule, Fig. 3A, B).

To supplement Wilhelmy balance experiments on spread films, aqueous dispersions of DPPC, CLSE, PPL, SP&PL, and N&PL were studied on an oscillating bubble apparatus at 37°C and high humidity (**Fig. 4**). Oscillating bubble experiments were done at a rate of 20 cycles/min, much higher than feasible with the Wilhelmy balance, and in the range of the respiratory system in vivo. In addition to increased cycling rate, oscillating bubble measurements with dispersed surfactants included the effects of adsorption as well as film dynamics, giving an overall assessment of activity incorporating the majority of the surface behaviors of functional lung surfactant (4, 28). Bubble results showed that dispersions of both CLSE and SP&PL at a concentration of 0.75 μmol/ml reached minimum surface tensions <1 mN/m (equivalent to maximum surface pressures >69 mN/m) under dynamic compression at rapid rate (Fig. 4). rCLSE, reconstituted from separated subfractions, had a timecourse of surface tension lowering on the bubble identical to CLSE as shown previously (1). Dispersions of PPL, which do not adsorb

TABLE 4. Surface pressures (π) at different areas during a single initial compression of monolayers of surfactant subfractions

Films	T (°C)	Cycling Rate	π (mN/m) at Molecular Areas (Å²/Molecule) 80	60	40	End
CLSE	23	10	14.7	35.2	46.7	48.0
SP&PL	23	10	13.0	33.7	46.2	51.2
N&PL	23	10	13.7	32.9	51.0	56.9
PPL	23	10	11.9	29.0	53.6	57.4
DPPC	23	10	5.5	9.6	69.3	72.0
CLSE	23	1.5	17.9	42.7	50.4	65.2
SP&PL	23	1.5	14.4	37.4	48.8	63.2
N&PL	23	1.5	16.4	41.0	58.2	63.7
PPL	23	1.5	15.5	39.8	60.5	62.9
DPPC	23	1.5	5.6	10.0	71.6	72.0
CLSE	37	10	25.3	44.6	46.8	47.0
SP&PL	37	10	23.3	43.4	46.1	47.1
N&PL	37	10	24.1	43.3	46.6	47.1
PPL	37	10	22.3	42.0	49.4	50.5
DPPC	37	10	20.1	40.3	53.8	55.4

At 23°C, compression was from an initial area of 120 Å²/phospholipid molecule to 27.6 Å²/molecule at end-compression. At 37°C, compression was from an initial area of 150 Å²/molecule to 34.4 Å²/molecule at end-compression. Cycling rate is in minutes per complete compression/expansion cycle. Surface pressure data are averages of at least four experiments with SEM < 5%.

nearly as well as protein-containing mixtures (data not shown), generated much higher minimum surface tensions on the bubble, as did dispersions of N&PL (Fig. 4).

DISCUSSION

This study has examined the dynamic surface pressure-area isotherms of interfacial films of the hydrophobic constituents of pulmonary surfactant in order to define more precisely their roles and importance in surfactant biophysics. Wilhelmy balance experiments with solvent-spread interfacial films identified specific effects from mixed surfactant phospholipids, neutral lipids, and proteins within the surface film itself, distinct from processes involving adsorption. The actions of the secondary surfactant phospholipids in improving respreading in interfacial films dynamically cycled into the collapse regime were particularly impressive (Tables 2, 3; Fig. 2). PPL films containing the complete mix of surfactant phospholipids had significantly enhanced respreading over DPPC alone for a variety of compression rates and film initial conditions at room and body temperature (Tables 2, 3). Additional improvements in film respreading were also found from the hydrophobic SP (Tables 2, 3; Fig. 2), complementing their known actions in enhancing lung surfactant adsorption (13–16, 29). These findings reinforce the perspective that functional pulmonary surfactant should be considered as a mixture of phospholipids and apoproteins, with interactions between them defining the behavior of the system as a whole.

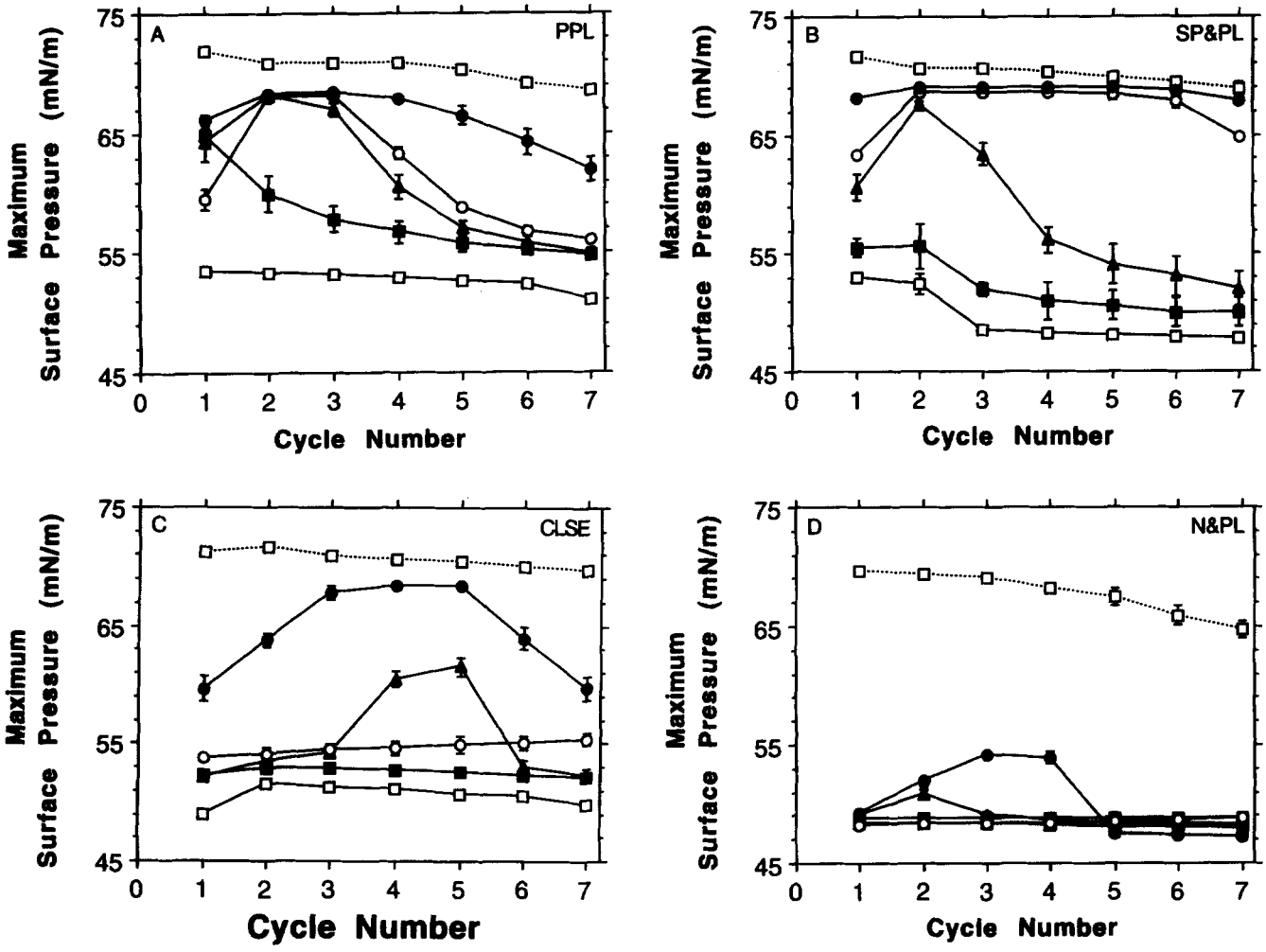

Fig. 3. Maximum surface pressures as a function of cycle number for films of CLSE and its subfractions. A: PPL; B: SP&PL; C: CLSE; D: N&PL. Dashed lines: 23°C; solid lines 37°C. Symbols designate different film initial concentrations and cycling rates as follows: (open squares), 60 Å²/phospholipid molecule films compressed at 10 min/cycle; (filled squares), 60 Å²/molecule films compressed at 1.5 min/cycle; (filled triangles), 30 Å²/molecule films compressed at 1.5 min/cycle; (open circles), 15 Å²/molecule films compressed at 5 min/cycle; and (filled circles), 15 Å²/molecule films compressed at 1.5 min/cycle. Maximum surface pressures >71 mN/m at 23°C or >69 mN/m at 37°C are equivalent to minimum surface tensions <1 mN/m.

TABLE 5. Effect of hydrophobic surfactant apoproteins on maximum surface pressure in cycled films with PPL and DPPC

Mixtures	Amount of SP (% *by wt*)	Maximum Surface Pressure (mNm) at Cycles 1	2	5	7
SP + PPL	0.0	64.8 ± 0.6	60.0 ± 1.5	55.8 ± 0.7	54.8 ± 0.5
SP + PPL	0.75	57.3 ± 0.7	53.2 ± 0.8	49.4 ± 1.1	48.6 ± 0.7
SP + PPL	1.30	55.5 ± 0.8	55.6 ± 1.9	50.6 ± 1.2	50.0 ± 1.2
SP + PPL	5.20	53.8 ± 1.5	57.5 ± 0.5	52.1 ± 1.7	50.2 ± 2.0
SP + DPPC	0.0	69.2 ± 0.1	69.2 ± 0.1	69.0 ± 0.1	69.0 ± 0.1
SP + DPPC	1.30	69.2 ± 0.3	69.1 ± 0.2	66.0 ± 0.6	64.1 ± 0.8
SP + DPPC	2.60	69.1 ± 0.1	69.1 ± 0.1	66.1 ± 0.5	63.2 ± 0.7
SP + DPPC	5.20	69.1 ± 0.1	68.3 ± 0.4	62.9 ± 0.7	59.9 ± 0.8

Mixtures of SP with PPL or DPPC in hexane–ethanol 9:1 (v/v) were spread initially to 60 Å²/phospholipid molecule in a Wilhelmy balance, and cycled continuously at 1.5 min/cycle at 37°C. Data are mean ± SEM for n = 4–10.

The majority of the constituents of lung surfactant are hydrophobic in nature and were represented in the CLSE and subfraction films studied. The primary surfactant constituent not present in our experiments was SP-A, the most polar of the three major surfactant apoproteins (14, 29). SP-A, together with SP-B and calcium, is required for the tubular myelin microstructure that facilitates the adsorption of phospholipids in endogenous lung surfactant (30, 31). However, the ability of surfactant extracts like CLSE to approach the overall surface activity of whole surfactant (3, 21) suggests that SP-A has less impact directly on the dynamic compression behavior of the surface film itself. The present studies of CLSE and its subfractions should thus encompass the major interactions in interfacial films of pulmonary surfactant.

The secondary phospholipids and hydrophobic SP in lung surfactant most likely increase respreading by affecting the structure and properties of film collapse structures generated in the immediate region of the interface during dynamic compression. Such structures may be multilayered on top of the underlying monolayer, or they may be located below the interface, or both. Ries and co-workers have demonstrated with electron microscopy that compressed fatty acid monolayers (32, 33) and DPPC films (34) generate suprasurface, multilayered structures during the collapse process. In addition, Sen et al. (35) have shown in freeze-fracture studies that surface films of CLSE and calf lung surfactant exhibit funnel-like aqueous phase structures containing phospholipids that are associated with the surface monolayer. This dependence on interfacial region structures, generated during dynamic compression of the surface film into the non-equilibrium collapse regime, makes respreading distinct from the equilibrium process of adsorption into a free interface.

Unsaturated phosphatidylcholines are the most prevalent secondary surfactant phospholipids (1, 6, 7, 11), and are probably responsible for a substantial part of the respreading improvements in PPL versus DPPC films (Tables 2, 3). However, other phospholipid classes such as anionic phospholipids in PPL may also have acted to improve respreading. Both the chain and headgroup regions of phospholipids influence their respreading behavior (8, 9, 17–19, 36, 37). Unsaturated phospholipids, with lower gel to liquid crystal transition temperatures than DPPC, are known to increase the respreading of this disaturated molecule in mixed films (8, 17–19). In addition, Liu et al. (36, 37) have demonstrated that the respreading of a series of phospholipid and phosphonolipid molecules with equivalent C16 saturated chains is strongly influenced by differences in headgroup molecular structure and ionization state. Chain-backbone junctional linkage group

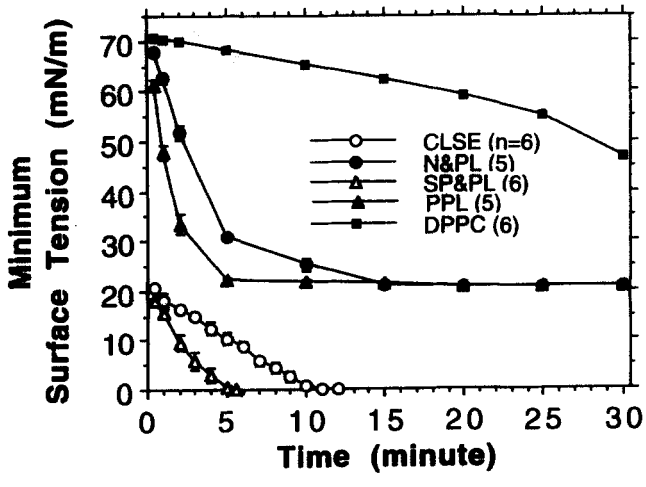

Fig. 4. Minimum surface tensions reached by dispersions of CLSE and its subfractions on an oscillating bubble surfactometer. Data are mean ± SEM for dispersions at 0.75 μmol/ml phospholipid concentration at 37°C and 20 cycles/min.

(ether, ester, amide), phosphate/phosphonate linkage, and N-headgroup structure (choline, phosphatidylethanolamine, etc.) were all found to affect dynamic respreading in surface-excess films (36, 37), correlated in part with molecular interactions involving headgroup hydrogen bonding, hydrophobicity, and charge (36–38). PPL contains molecules with a spectrum of headgroup sizes, charge, and hydrophobicity, and this may be important along with chain-region differences for the respreading of this complex mixture.

Mechanistically, secondary lung surfactant phospholipids and hydrophobic proteins might improve respreading by direct incorporation into film collapse aggregates, altering their size, fluidity, cohesiveness, or degree of association with the interfacial film. Even if not fully miscible, unsaturated phospholipids with increased chain fluidity and molecular spacing would increase disorder in film collapse phases for PPL compared to DPPC, potentially improving the respreading of adjacent clusters of saturated molecules. In addition to chain-mediated effects, electrostatic and hydrogen bonding interactions between phospholipid headgroups could affect the size or character of collapse structures in films of PPL versus DPPC (36, 37). Hydrophobic SP are also known directly to affect phospholipid aggregates and bilayers. By virtue of their hydrophobic structure, these molecules can intercalate in phospholipid bilayers (14, 29, 39–42) and influence aggregate structure in the aqueous phase (43). In addition, hydrophobic SP could influence film pressures and respreading by changing the composition of lipids within the interfacial film during compression, such as by altering DPPC content through selective squeeze-out of different film components during compression.

The decreased maximum surface pressure in SP&PL films compared to PPL films for initial spreading to 150, 60, and 30 Å²/phospholipid molecule (Tables 4, 5; Fig. 3A, B) argues against a major stabilizing role for apoproteins in lung surfactant films as suggested by Baatz et al. (39, 40). However, stabilizing effects may have been present in SP&PL films spread initially to surface-excess concentration (15 Å²/molecule, Fig. 3B vs. 3A). Longo et al. (44) also found that SP-B increased surface pressures near the collapse regime in palmitic acid films. The ability of the hydrophobic SP to achieve beneficial effects on respreading without compromising surface tension lowering at high film concentrations is important, as lung surfactant must maintain a balance of components giving exceptionally low surface tensions together with good respreading. In order to lower surface tension into the range of 1–10 mN/m at 37°C (surface pressures above 60 mN/m), lung surfactant films must contain a significant DPPC content and be compressed dynamically to low molecular areas (2–5). Several fluid phospholipids that respread better than DPPC also lower surface tension much less effectively in dynamically compressed films (8, 17–19, 45, 46). However, this effect was not present with the hydrophobic SP in concentrated films, nor was it a major problem with the complete mix of surfactant phospholipids in PPL, which were particularly active in enhancing respreading. PPL films at 37°C did have lower maximum pressures compared to DPPC, but the effect was relatively mild and maximum π increased during rapid cycling at high concentration (Fig. 3).

Because dynamic film behavior is a function of cycling rate and film initial condition in addition to temperature and concentration, isotherm measurements were made for a range of film initial concentrations and compression rates. In addition, since physiological rates could not be reached on the Wilhelmy balance, and its large subphase (1 liter) contained no surface active material, oscillating bubble experiments were done to define overall surface tension lowering ability under more physiologically relevant conditions (Fig. 4). Dispersions of CLSE and SP&PL at a subphase concentration of 0.75 μmol phospholipid/ml both generated minimum surface tensions <1 mN/m on the oscillating bubble at 20 cycles/min (Fig. 4). Wilhelmy balance studies with CLSE dispersed in the subphase at 0.02 μmol phospholipid/ml also showed that adsorbed films could generate minimum surface tensions below 1 mN/m for compression at 1.5 min/cycle at 37°C (data not shown). PPL dispersions had higher minimum surface tensions on the oscillating bubble (Fig. 4), almost certainly reflecting reduced adsorption compared to CLSE. A number of previous studies have found that the adsorption of complex synthetic phospholipid mixtures containing DPPC is significantly less than that of CLSE or whole surfactant (e.g., 12, 13, 47 plus 3, 46 for review). The slightly longer timecourse of surface tension lowering for CLSE versus SP&PL on the oscillating bubble (Fig. 4) was apparently due to the generally detrimental action of neutral lipids on this variable.

Our finding that the surface active properties of lung surfactant phospholipids are significantly different from DPPC has ramifications not only for natural surfactant activity, but also for the development of exogenous lung surfactants as well. Previous studies of exogenous surfactants containing synthetic phospholipids mixed with purified or cloned lung surfactant apoproteins have used model mixtures with a high DPPC content (typically 80% or greater) (3, 21). Relatively little systematic attention has been paid to the secondary phospholipid content of these exogenous surfactants, which generally only roughly approximate PPL. The significant improvements in dynamic respreading in PPL films, and the very different overall isotherm behavior of these mixed phospholipids compared to DPPC under a variety of experimental conditions, suggest that future studies should address more completely the activity of PPL or closely related synthetic phospholipid mixtures as components in new exogenous lung surfactants.

In summary, our dynamic studies on films of the hydrophobic constituents of lung surfactant show the following. *1)* PPL films, with the complete mix of surfactant phospholipids, had greatly improved dynamic respreading compared to DPPC. Dynamic π-A isotherms of PPL films for a variety of initial concentrations and rates were shifted substantially toward CLSE and away from DPPC. Respreading was increased further in SP&PL versus PPL films, indicating an additional contribution of hydrophobic SP to this film behavior. Neutral lipids also increased respreading, but the surface tension lowering ability of N&PL films at body temperature was consistently worse than for CLSE, SP&PL, or PPL. *2)* Maximum π was increased slightly in SP&PL versus PPL films spread to 15 Å^2/molecule, suggesting that SP improved stability at high film concentrations, although maximum pressures were lower in SP&PL versus PPL films spread initially to $\leq$60 Å^2/molecule. *3)* Dynamic π-A isotherms for CLSE films reflected their composition of mixed phospholipids, neutral lipids, and hydrophobic SP. Maximum π was generally slightly lower in CLSE versus SP&PL films, and respreading was slightly higher, due to the additional presence of neutral lipids in the surfactant extract. However, at 37°C, surface-excess films of SP&PL and CLSE both reached high maximum π > 68 mN/m (minimum surface tensions <2 mN/m) during dynamic compression at 1.5 min/cycle on the Wilhelmy balance, and adsorbed CLSE and SP&PL films had minimum surface tensions <1 mN/m when compressed on the oscillating bubble at physiologic cycling rate.

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