Interaction of pulmonary surfactant protein A with dipalmitoylphosphatidylcholine and cholesterol at the air/water interface

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Abstract Interaction of pulmonary surfactant protein A (SP-A) with pure and binary mixed dipalmitoylphosphatidylcholine (DPPC) and cholesterol (3.5 wt%) at the air/saline, 1.5 mm $CaCl_2$ interface was investigated using a rhomboid surface balance at 37°C. Surface tension–area isotherms were measured to access the surface active properties of the monolayers. The organization of DPPC and cholesterol in DPPC and DPPC/cholesterol mixed monolayers with or without SP-A at equilibrium surface tension (≈23 mN/N) was revealed by autoradiographs of Langmuir-Blodgett (L-B) films deposited from [^{14}C]DPPC or [^{14}C]cholesterol-labeled monolayers. The results showed that SP-A can interact with the polar head groups of DPPC monolayers and aggregate DPPC molecules. SP-A decreased the surface area reduction required for DPPC monolayers to achieve near zero surface tension from 30 to 25% of the area at equilibrium. SP-A also reduced the collapse surface tension of pure cholesterol from 27 to 23 mN/m. DPPC and cholesterol formed homogeneous mixed monolayers when both were dissolved in the spreading solvent prior to spreading, while separate cholesterol-rich domains appeared when DPPC and cholesterol were spread successively. Cholesterol resisted squeeze-out from either mixed monolayer through compression. Although SP-A could not promote the squeeze-out of cholesterol from homogeneous mixed monolayers, it facilitated that of cholesterol domains especially when SP-A had first interacted with DPPC. These results indicate that pulmonary surfactant protein A facilitates the squeeze-out of cholesterol domains from mixed monolayers by condensing DPPC and limiting lateral interactions of DPPC with cholesterol domains.—**Yu, S-H., and F. Possmayer.** Interaction of pulmonary surfactant protein A with dipalmitoylphosphatidylcholine and cholesterol at the air/water interface. *J. Lipid Res.* 1998. **39:** 555–568.

Supplementary key words air/water interface • dipalmitoylphosphatidylcholine • monolayer • cholesterol • L-B film • pulmonary surfactant • surface tension • surfactant-associated protein A

The alveolar epithelium is covered with a thin layer of pulmonary fluid carrying a film of pulmonary surfactant at the air/water interface. Dipalmitoylphosphatidylcholine (DPPC) is the principal component of pulmonary surfactant that is capable of reducing the surface tension of alveoli to near zero by compression, thereby preventing collapse of the lung during expiration (1–4).

Pulmonary natural surfactant, obtained through centrifugation of lung lavage, is a complex mixture of about 90% lipids and 10% proteins (5, 6). The lipids are composed of 93–96% phospholipid (PL) and 4–7% neutral lipid (NL) containing mainly cholesterol. The surfactant-associated proteins (SP-) consist of about 80% hydrophilic protein A (SP-A), and 20% two small hydrophobic proteins SP-B and SP-C (7). SP-A is a glycoprotein of 35 kDa with four structural domains (8): a disulfide-forming amino terminal, a collagen-like domain, a short hydrophobic neck region, and a carbohydrate-binding carboxyl-terminal domain. In the presence of calcium, SP-A aggregates PL vesicles (9) and binds to DPPC (10). It plays important roles in the biological and biophysical functions of the pulmonary surfactant (11).

In the past we have prepared three types of bovine pulmonary surfactant in our laboratory: bovine natural surfactant, bovine lipid extract surfactant [bLES (chol)], and lipid extract surfactant without neutral lipid (bLES) (12). bLES(chol) was produced by chloroform–methanol extraction of natural surfactant; it contains all the components of natural surfactant except SP-A. When neutral lipid was removed from

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; L-B film, Langmuir-Blodgett film; NL, neutral lipid; PL, phospholipid; bLES, bovine lipid extract surfactant; SP-, surfactant-associated protein.

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bLES(chol) by acetone precipitation, we obtained bLES which contains phospholipids SP-B and SP-C. Content of neutral lipid (mainly cholesterol) in bLES(chol) is rather low, only about 4 wt% of total lipids (6).

Pulmonary surfactant is synthesized in alveolar type II cells and secreted into the subphase that covers the alveolar epithelium (11). It has become evident that the surface film formed from a dispersion of pulmonary surfactant is composed of a monolayer and a monolayer-associated surfactant layer (12–15). At physiological temperatures, DPPC alone adsorbs very slowly to the air/water interface. However, a rapid adsorption of DPPC from [^{14}C]DPPC-labeled pulmonary surfactant dispersion was observed, especially in the presence of SP-A and cholesterol (12). We have also observed profound effects of SP-A and cholesterol on the accumulation and organization of DPPC in surface films by preparing Langmuir-Blodgett (L-B) films (12). Nonetheless, in those studies, we were unable to distinguish between monolayer and monolayer-associated surfactant layers.

Although the same surfactant components were observed in spread and adsorbed monolayers using a simple surfactant model consisting of DPPC and SP-C (16), it is not known whether all the components of pulmonary surfactant are adsorbed into the monolayer or how the non-DPPC components are eliminated during compression. Several studies (17–20) have demonstrated that cholesterol cannot be squeezed out readily from mixed monolayers through repeated compression. Previously using [^{14}C]cholesterol we have shown that cholesterol could be readily transported to the surface in bLES(chol) dispersions but not by itself, and SP-A suppressed the transport of cholesterol to the surface (19). We have also observed that bLES(chol) or cholesterol-supplemented bLES requires more pulsations, in a pulsation surfactometer, to reach near zero surface tension than bLES does. Moreover, natural surfactant, which contains SP-A and cholesterol in addition to PL, SP-B, and SP-C, has a greater surface tension-lowering ability than either bLES(chol) or bLES, both of which lack SP-A (21). These results indicated that SP-A facilitates the squeeze-out of cholesterol from mixed monolayers. However, these earlier experiments were all performed with dispersions that formed more than monolayers at the air/water interfaces. In the present study we investigate the interactions between and among SP-A, DPPC, and cholesterol in spread monolayers. We have built a rhomboid surface balance to examine the changes in surface area reduction of DPPC monolayer from equilibrium to near zero surface tension, in the presence and absence of SP-A and/or cholesterol. We also investigate the effect of SP-A on the organization of DPPC as well as cholesterol in pure DPPC and DPPC/cholesterol mixed monolayers by examining autoradiographs of [^{14}C]DPPC or [^{14}C]cholesterol-labeled monolayers at equilibrium surface tension using the L-B film deposition technique (22).

It should be noted that "squeeze-out" used in the present studies implies the loss of lipids by compression from the monolayer but not necessarily from the surface film. Squeezed out lipids could remain associated with the surface monolayer below the interface. Such squeeze-out lipids could re-enter the surface as the film was expanded.

MATERIALS AND METHODS

Materials

DPPC and cholesterol were purchased from Sigma Chemical Co. (Mississauga, Ontario, Canada). Dipalmitoyl-1-[^{14}C]phosphatidylcholine and [26-^{14}C]cholesterol were obtained from New England Nuclear (Markham, Ontario, Canada). Purity of labeled and nonlabeled chemicals was confirmed by thin-layer chromatography. Concentrations of ^{14}C were verified with a scintillation counter (Beckman LS6000 IC). All reagents and other chemicals (analytical grade) were from BDH Inc. (British Drug House). Distilled water purified through a Millipore Milli-Q four-cartridge system was used in all experiments.

Preparation of surfactant-associated protein (SP-A)

SP-A was prepared according to a previously published method (23). Briefly, 10 mg bovine natural surfactant was dispersed in 1 ml 6 m urea/0.05% trifluoroacetic acid, centrifuged, and filtered. Approximately 200 μl of the clear solution was applied to a 30 × 2.8 cm C_{18} reverse-phase column (Waters Scientific). SP-A was eluted with a linear gradient of 2-propanol in 0.05% trifluoroacetic acid at a flow rate of 0.8 ml/min. SP-A fractions were pooled, trifluoroacetic acid was evaporated under N_2, and the protein was lyophilized. Quantity of SP-A was determined by the method of Lowry et al. (24). Purity of SP-A was estimated by sodium dodecyl sulfate–urea–polyacrylamide gel electrophoresis.

Effect of SP-A on the surface tension of DPPC monolayers

The surface tension of monolayers was measured with the Wilhelmy plate technique as described previously (25). A 5-mm wide sand-blasted platinum plate was dipped into 5 ml saline, 1.5 mm $CaCl_2$, in a round Teflon dish (2.5 cm dia. × 2 cm depth), resting in a

temperature-regulated sand bath (37 ± 1°C) enclosed in a temperature-controlled box (37 ± 0.5°C). Surface tension was monitored with a TSAR 1 computer-controlled readout (TECH-SER, Inc., Torrance, CA). DPPC, 0.5 mg/ml in hexane–methanol 95:5, was spread on the air/water interface to the desired surface tension. After 15 min of solvent evaporation, a 10-μl SP-A solution in 0.4 mg/ml water–2-propanol 2:1 was applied onto the DPPC monolayer. SP-A (10 μl) was also applied onto the air/saline, 1.5 mm $CaCl_2$ interface in the absence of the DPPC monolayer. In another set of experiments, a sample of 50 μg SP-A in 0.5 mg/ml saline, 1.5 mm $CaCl_2$ was injected into the subphase (5 ml saline, 1.5 mm $CaCl_2$), with or without DPPC monolayers, through an injection hole. Samples were stirred slowly at 30 rpm. A sample of 10 μl SP-A in 0.5 mg/ml saline, 1.5 mm $CaCl_2$ was also applied onto the interface with or without DPPC monolayer.

Surface tension–surface area measurements

In order to eliminate the possible leakage of surfactant material from the edges of the trough, we built a modified Wilhelmy surface balance with a rhomboid compression frame suspended in the trough. The design of this apparatus is similar to that of Schoedel, Slama and Hansen (26). Both trough and rhomboid were made of Teflon. The trough had an area of 10 × 10 cm^2 with 2 cm depth. The rhomboid was 5 cm long on each side and 1.5 cm in height, leaving a 5 mm distance between the bottom of rhomboid and trough. One corner of the rhomboid had a short arm secured on one rim of the trough; the opposite corner had a long arm connected to a synchronous stepping motor that was controlled by a computer for performing dynamic compression and expansion. The long arm traveled through a guide bracket on the rim of the trough. The movements of the rhomboid and the surface tension at the air/water interface were recorded with an XY recorder (SE 120 Waters Scientific). Percentage changes in area from equilibrium to near zero surface tension were obtained from an area–distance graph plotted by the computer. In a typical experiment, 120 ml saline, 1.5 mm $CaCl_2$ was poured into the trough sitting on a temperature-regulated hot plate (37 ± 0.5°C) enclosed within a temperature-controlled box (37 ± 0.5°C). A 5-mm wide platinum plate was dipped into the subphase and the surface tension was monitored as described above. A sample of DPPC, cholesterol, or DPPC/cholesterol mixture in hexane–methanol 95:5 (1 mg/ml) was spread on the air/water interface to the desired surface tension. Dynamic compression/expansion was initiated after 15 min of solvent evaporation; six cycles of compression/expansion between maximum and minimum areas of 24 and 8 cm^2 (compression ratio of 3:1) at a speed of 1.5 min/cycle were performed. In the experiments including SP-A, a 20 μl solution of 0.4 mg/ml SP-A in water–2-propanol 2:1 was applied on the interface either before or after formation of the lipid monolayer. In the case of applying SP-A first, lipid was spread immediately after the application of SP-A. When lipid was spread prior to SP-A, SP-A was added after a 15-min pause for solvent evaporation. In either case, dynamic cycling commenced 30 min after sample application was completed. The initial surface tension of DPPC was 30–31 mN/m and cholesterol was 3.5 wt% in all DPPC/cholesterol mixed monolayers. Similar experiments were performed by applying 50 μl SP-A (0.5 mg/ml saline, 1.5 mm $CaCl_2$) onto pure DPPC monolayers (30–31 mN/m).

Langmuir-Blodgett film deposition and autoradiography

L-B films of monolayers in the rhomboid surface balance were usually prepared at equilibrium surface tension. Surface tension was monitored as described above. A clean microscope glass cover slip (Fisher Scientific) was lowered into the subphase (saline, 1.5 mm $CaCl_2$). Sample applications were performed as in dynamic cycling studies. A ^{14}C-labeled sample of DPPC, cholesterol, or DPPC/cholesterol mixture in hexane–methanol 95:5 was spread on the air/water interface to the desired surface tension. In most cases, the initial surface tension of DPPC was 30–31 mN/m and cholesterol was 3.5 wt% of DPPC. When SP-A was present in the experiment, 20 μl of 0.4 mg/ml SP-A in water–2-propanol 2:1 was applied onto the interface either before or after formation of the lipid monolayer. Thirty minutes after complete spreading, the monolayer was compressed slowly at 0.8 mm/min. The glass plate was lifted slowly when the surface tension reached the equilibrium value (23–24 mN/m), and the surface tension was maintained constant throughout the deposition process. When a film was deposited at near zero surface tension, the monolayer was compressed from the initial surface tension to near zero at 60 mm/min. The compression speed was then reduced to 0.8 mm/min and the glass plate was raised slowly at constant surface tension.

Monolayers of DPPC at the interface of saline, 1.5 mm $CaCl_2$ in a round Teflon dish (2.5 cm dia × 2 cm depth) were also deposited on glass plates without compression. In a typical experiment, a sample of [^{14}C] DPPC-labeled DPPC was spread to about 24 mN/m, then 50 μg SP-A (0.5 mg/ml in saline, 1.5 mm $CaCl_2$) was injected into the subphase through the injection hole after 15 min of solvent evaporation. SP-A was allowed to diffuse for 30 min without stirring. The glass plate, which was lowered into the subphase prior to spreading of DPPC, was raised at 1 mm/min.

The specific radioactivity of all samples was 2.5 μC_i/mg total lipid. Consequently the specific radioactivity of [^{14}C]cholesterol is about thirty times that of [^{14}C]DPPC. The area of deposition was 1 × 1 cm^2 and the experimental temperature was maintained at 37 ± 0.5°C unless specified otherwise. Each experiment was performed 3–5 times. Autoradiographs of L-B films were prepared by exposing the plates to X-ray film for 50 h at 4°C. The figures show autoradiographs of the entire 1 × 1 cm^2 glass plates.

RESULTS

Interaction of SP-A with DPPC

The Wilhelmy plate technique was used to measure the effect of SP-A on the surface tension at the air/saline, 1.5 mm $CaCl_2$ interface with or without DPPC monolayers. **Figure 1** shows that the surface tension of a clean surface of saline, 1.5 mm $CaCl_2$ (70 mN/m) was not affected either by injecting SP-A (in saline, 1.5 mm $CaCl_2$) into the subphase (A), or applying SP-A (in either saline, 1.5 mm $CaCl_2$ or water–2-propanol 2:1) on the interface (B). These results indicate that SP-A is not surface active; it could not adsorb to or remain at the clean interface. Although application of pure water–2-propanol 2:1 on a DPPC monolayer did not affect the surface tension, when SP-A in water–2-propanol 2:1 was applied onto a DPPC monolayer (Fig. 1B, 30 or 50 mN/m initially), the surface tension of the monolayer was reduced. The higher the original surface tension, the greater the reduction in surface tension. Surface tension decreased swiftly as soon as SP-A was applied and gradually increased to a constant value lower than the original. These results indicate that when SP-A in water–2-propanol 2:1 was applied onto a DPPC monolayer, a small portion of SP-A remained at the surface, while the rest of SP-A gradually moved through the monolayer into the subphase. Some of this SP-A may remain associated with DPPC monolayer. However, the surface tension of DPPC monolayers (30 or 50 mN/m) was not altered by injecting SP-A (in saline, 1.5 mm $CaCl_2$) into the subphase (Fig. 1A). Nor was the surface tension affected by applying SP-A in saline, 1.5 mm $CaCl_2$ onto the DPPC monolayer, indicating that all the SP-A diffused through the monolayer into the subphase. These results show that 2-propanol could delay the diffusion of SP-A through an interface containing a DPPC monolayer (but not a clean interface) into the subphase.

Figure 2 displays autoradiographs of L-B DPPC monolayers deposited on glass plates. In Figs. 2A and 2C, the L-B films were made in a round Teflon dish without compression, while in 2B and 2D they were made in the rhomboid surface balance at constant surface tension throughout the deposition process. In Fig. 2A, the surface tension increased from the initial 23–24 mN/m (equilibrium value) to about 35 mN/m as a result of DPPC removal. A few small aggregates can be seen, suggesting that the initial film may be slightly in surface excess. Fine homogeneous particles can be seen throughout the plate despite the fact that the surface tension was not constant during the deposition process. This may be a phenomenon of the Morangoni effects (27). Figure 2B exhibits a homogeneous DPPC

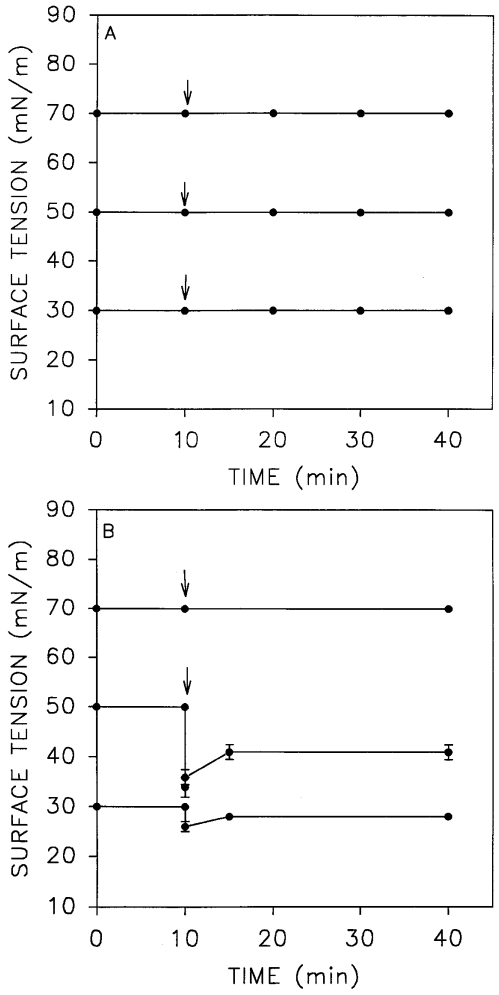

Fig. 1. Surface active properties of SP-A and its interaction with DPPC monolayers at 37°C. A: 50 μg SP-A in saline, 1.5 mm $CaCl_2$ was injected into 5 ml subphase (saline, 1.5 mm $CaCl_2$) in a round Teflon dish under a clean surface (70 mN/m) or monolayers of DPPC (30 and 50 mN/m). Dispersions were stirred at 30 rpm. B: 10 μl of 0.4 mg/ml SP-A in water–2-propanol 2:1 (4 μg) was applied on the air/saline, 1.5 mm $CaCl_2$ interface with (30 and 50 mN/m) or without (70 mN/m) DPPC monolayers.

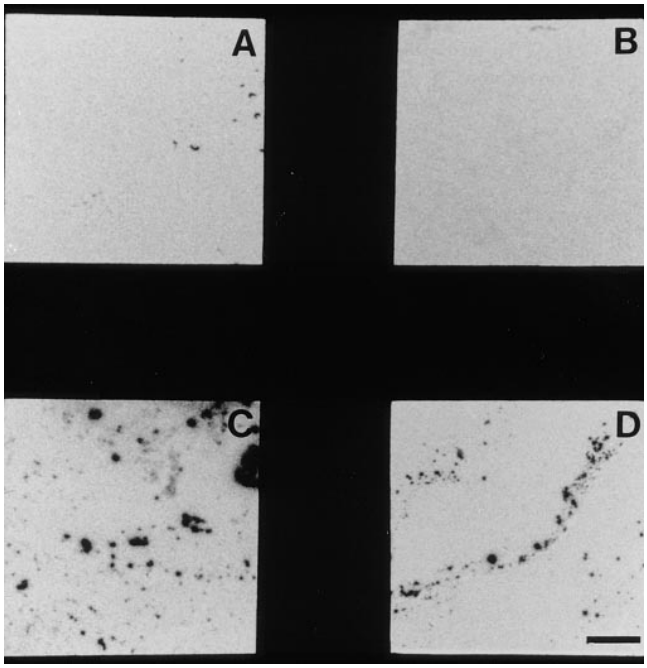

Fig. 2. Autoradiographs of L-B films showing interaction of DPPC and SP-A. L-B films were deposited from [^{14}C]DPPC-labeled DPPC monolayers at 37°C with: A, an initial surface tension of 23–24 mN/m in a round Teflon dish, L-B film was deposited without compression; B, an initial surface tension of 30–31 mN/m in the rhomboid surface balance and compressed to 23–24 mN/m, L-B film was made at the constant surface tension of 23–24 mN/m; C, an initial surface tension of 24–25 mN/m in a round Teflon dish, 50 μg SP-A was injected into the 5 ml subphase through the injection hole. The L-B film was deposited 30 min after injection of SP-A without compression; D, an initial surface tension of 30–31 mN/m in the rhomboid surface balance, 8 μg SP-A (20 μl 0.4 mg/ml in water–2-propanol 2:1) was applied on DPPC monolayer, the film was compressed to 23–24 mN/m and deposited on the plate at the constant surface tension. Experimental details as described in the text. The entire autoradiographs of 1 × 1 cm^2 glass cover slips are shown. The bar indicates 2 mm in all graphs.

monolayer without aggregation. It was deposited at a constant surface tension of 23–24 mN/m from a DPPC monolayer having an initial surface tension of 30–31 mN/m. In Fig. 2C, the L-B film was deposited, after injecting SP-A into the subphase, as the surface tension rose from 24–25 mN/m to 37–38 mN/m. Heterogeneous aggregates of DPPC can be seen, indicating that SP-A diffused to the surface and interacted with the polar head groups of DPPC. When SP-A (in water–2-propanol 2:1) was applied onto a DPPC monolayer with an initial surface tension at 30–31 mN/m, followed by compressing the film to the equilibrium value of 23–24 mN/m for film deposition, a localized aggregation of DPPC can be seen (Fig. 2D). Aggregation of DPPC also occurred when SP-A in an aqueous medium (saline, 1.5 mm $CaCl_2$) was applied onto DPPC monolayers (30–31 mN/m, results not shown), the surface tension was not altered by the addition of SP-A. This shows that SP-A, either injected under the monolayer or applied from above, interacts with the monolayer resulting in DPPC aggregation.

Table 1 summarizes the surface properties of pure DPPC and DPPC/cholesterol mixed monolayers in the presence and absence of SP-A. **Figure 3** shows a typical dynamic surface tension–surface area isotherm of DPPC and DPPC plus SP-A which was dissolved in water–2-propanol 2:1. It was found that at 37°C, the surface area of a pure DPPC monolayer had to be compressed by about 30% in order to lower the surface tension from the equilibrium value to near zero. SP-A facilitated DPPC monolayers to achieve the same surface tension reduction with only about 25% area reduction (Table 1, rows 2–4), likely owing to the aggregation of DPPC induced by SP-A. As a result, fewer DPPC molecules were squeezed out from the monolayer during compression. A distinct plateau, an indication of the loss of monolayer material during compression, was observed around 23–24 mN/m of compression cycle 1 for the DPPC monolayer in the absence of SP-A (Fig. 3A). This plateau was diminished in the presence of SP-A (Fig. 3B). This could be attributed to the aggregation of DPPC by SP-A, thereby causing a decrease in the loss of DPPC molecules from the interface. It was also noted that similar isotherms were obtained whether SP-A was applied before or after formation of the DPPC monolayer (Fig. 3B). As the application of SP-A (in water–2-propanol 2:1) on a clean interface (before the formation of DPPC monolayer) did not affect the surface tension, this observation also suggests that SP-A affected the surface activity of DPPC monolayer by interacting mainly with DPPC head groups. SP-A applied after DPPC monolayer formation had a modest effect on surface tension (≈2 mN/m), suggesting only small amounts of SP-A remained extended across the interface. DPPC monolayers with and without SP-A exhibited a moderate elevation of γ_{max} and γ_{min} after six cycles of compression and expansion. Most of the increase in surface tension occurred during the first cycle when the films were over-compressed at the near zero surface tension. The presence of DPPC overlapping layers on the surface at the collapse surface pressure (near zero surface tension) has been shown in electron micrographs and such DPPC layers were unable to reenter the interface completely by expansion (28). This could explain the significant changes in isotherms observed between cycle 1 and cycle 2. A similar surface area reduction (≈25%) was required to achieve the near zero surface tension when SP-A in an aqueous medium (saline, 1.5 mm $CaCl_2$) was applied onto DPPC

TABLE 1. Interaction of SP-A with DPPC and cholesterol spread monolayers at the air/water interface

Monolayers[a]	Initial Surface Tension[b]	Min & Max Surface Tension at[c]						Reduction of Surface Area (%) Required from Equilibrium γ to Near Zero γ[d]
		Cycle 1		Cycle 2		Cycle 6		
		γ_{min}	γ_{max}	γ_{min}	γ_{max}	γ_{min}	γ_{max}	
	mN/m							*%*
1. DPPC	30–31	0.1 ± 0.1	55.0	2.0 ± 0.3	57.8	2.9 ± 0.3	59.0	29.8 ± 1.1
2. DPPC + SP-A	30–31 → 28–29	0.1 ± 0.1	55.8	1.9 ± 0.3	58.0	2.8 ± 0.5	59.0	25.0 ± 0.4
3. SP-A + DPPC	30–31	0.1 ± 0.1	55.5	1.3 ± 0.5	57.5	2.4 ± 0.4	58.5	24.5 ± 0.9
4. DPPC + SP-A	30–31	0.1 ± 0.1	56.5	1.6 ± 0.3	59.0	2.5 ± 0.5	60.0	25.3 ± 0.8
5. DPPC + CHOL(m)	26–27	2.2 ± 0.7	54.7	2.9 ± 0.7	60.7	6.8 ± 0.5	65.8	>60
6. DPPC + CHOL	30–31 → 26–27	1.5 ± 0.4	48.0	3.6 ± 1.0	54.7	7.5 ± 0.9	64.7	>60
7. DPPC + CHOL(m) + SP-A	26–27 → 24–25	1.4 ± 0.4	54.8	2.4 ± 0.5	60.5	6.2 ± 0.4	64.0	>60
8. SP-A+DPPC+CHOL(m)	26–27	1.5 ± 0.5	56.5	3.2 ± 0.5	61.0	7.5 ± 0.9	64.0	>60
9. DPPC+CHOL+SP-A	30–31 → 26–27 → 24–25	0.1 ± 0.1	49.8	3.4 ± 1.3	56.3	6.8 ± 1.6	62.8	41.0 ± 2.4
10. DPPC+SP-A+CHOL	30–31 → 28–29 → 24–25	0.1 ± 0.1	51.4	3.8 ± 0.7	59.2	8.0 ± 0.7	64.8	25.0 ± 0.9

Six successive cycles of compression/expansion at air/saline, 1.5 mm $CaCl_2$ interface were performed between maximum and minimum surface areas of 24 and 8 cm^2 (33.3% of the maximum area) at 1.5 min/cycle and 37°C.

[a]Composition of a monolayer is expressed in the order of the successive spreading of each component except for DPPC+CHOL(m) which was mixed in spreading solvent prior to spreading.

[b]Initial surface tension of DPPC in all samples was 30–31 mN/m. Successive spreading of SP-A and/or cholesterol decreased surface tension slightly as indicated by arrows. All monolayers of DPPC plus cholesterol contain 3.5 wt% cholesterol. Twenty μl SP-A (0.4 mg/ml in water–2-propanol 2:1) was applied in all samples containing SP-A except for row 4 where 50 μl of 0.5 mg/ml SP-A in saline, 1.5 mm $CaCl_2$ was used.

[c]Data are mean ± SE for $n \geq 4$ with SE < 4% for γ_{max}.

[d]Indicates surface area reductions for cycle 1. Equilibrium surface tension was 23 mN/m. Monolayers of rows 5–8 reached low surface tensions only at the maximum compression. *P* values (two-tailed *t*-test) for the effect of SP-A on required area reduction from equilibrium to near zero surface tension are: $P_{1,2} < 0.05$, $P_{1,3} < 0.05$, and $P_{1,4} < 0.05$, while no significant difference was observed due to the addition of cholesterol: $P_{2,10} > 0.05$.

monolayers. In this case the surface tension (30–31 nM/m) was not altered by the addition of SP-A (Table 1, row 4). This indicates that SP-A can affect the surface property of DPPC monolayers without interacting with the hydrocarbon moieties of DPPC.

Interaction of cholesterol with DPPC

We used DPPC supplemented with 3.5 wt% cholesterol, which is similar to the cholesterol content in bLES(chol), throughout this study except for one L-B film (see Fig. 5E). DPPC/cholesterol mixed monolayers were formed either simultaneously with both lipids dissolved in the spreading medium or successively with two separate lipid solutions. **Figure 4** shows the dynamic compression/expansion curves of DPPC and cholesterol mixed monolayers. The results show that whether the DPPC/cholesterol mixed monolayers were formed simultaneously or successively, the mixed monolayers could barely reach very low surface tension with the maximum compression of 66% surface area. Both γ_{max} and γ_{min} increased more markedly with successive compression/expansion cycles than without cholesterol. Again the largest effect was observed in the first cycle.

Figure 5 illustrates the organization of [^{14}C]DPPC and [^{14}C]cholesterol in L-B films deposited from DPPC/cholesterol mixed monolayers at 23–24 mN/m. Figure 5A shows that [^{14}C]DPPC was distributed evenly in the DPPC/cholesterol mixed monolayer formed simultaneously, along with some small aggregates (compare Fig. 5A with Fig. 2B). When the mixed monolayer was formed successively (Fig. 5B), [^{14}C]DPPC was found in larger heterogeneous aggregates. Figures 5C and 5D depict a very different distribution of [^{14}C]cholesterol in DPPC/cholesterol mixed monolayers when they were formed simultaneously (Fig. 5C) and successively (Fig. 5D). A homogeneous distribution of [^{14}C]cholesterol with small aggregates is observed in Fig. 5C, suggesting that DPPC and cholesterol had dissolved completely in the spreading solvent prior to spreading on the air/water interface. Figure 5D shows that when spread separately DPPC and cholesterol are immiscible, forming separate cholesterol domains. The cholesterol-containing areas appear larger than the relative concentration (3.5 wt%), suggesting that these domains are cholesterol-rich rather than pure cholesterol. The high specific radioactivity of [^{14}C]cholesterol may also contribute to this appearance. Figure 5E reveals an organization of [^{14}C]cholesterol similar to that in Fig. 5D, even with a higher content of cholesterol (10 wt% or 20 mol%). When the film was spread and deposited at 42.5°C a homogeneous L-B film was obtained (Fig. 5F) even with successive spreading, indicating that cholesterol could mix homogeneously with DPPC when both are in the fluid state regardless of the monolayer-forming process.

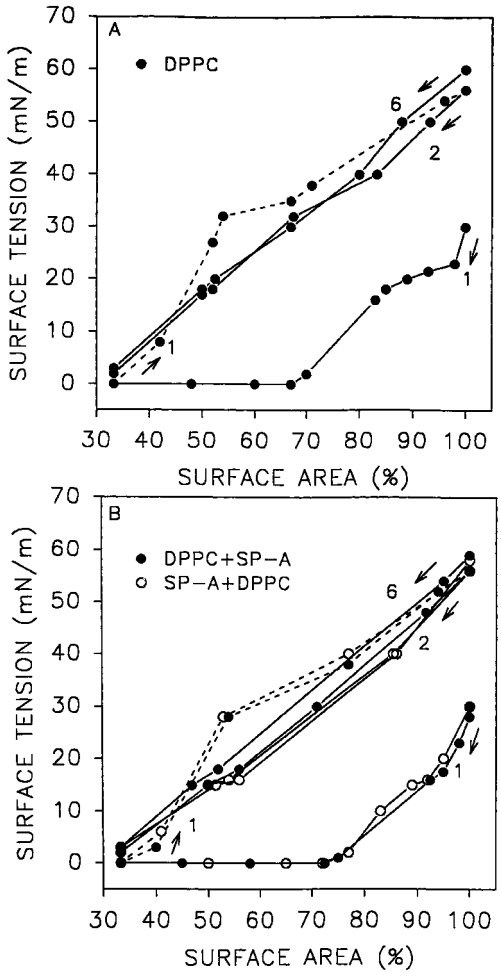

Fig. 3. Representative dynamic compression/expansion cycling of DPPC surface active films at 37°C. Experiments were carried out in the rhomboid surface balance as described in the text. SP-A was dissolved in water–2-propanol 2:1. Six cycles were performed at 1.5 min/cycle. Solid lines are compression curves and the dashed lines are expansion curves. A: surface tension–surface area isotherms of DPPC monolayer; B: surface tension–surface area isotherms of DPPC monolayer in the presence of SP-A; SP-A was applied before (○—○) or after (●—●) formation of the DPPC monolayer.

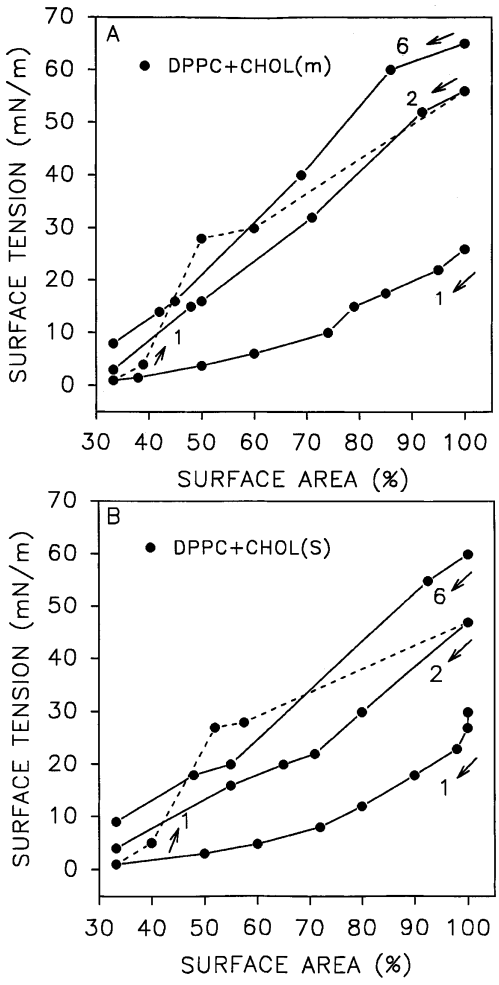

Fig. 4. Representative dynamic compression/expansion cycling of mixed DPPC/cholesterol surface active films at 37°C. Experimental details as in Fig. 3. A: DPPC and 3.5 wt% cholesterol were dissolved together in hexane–methanol 95:5 (1 mg/ml) and spread on air/saline, 1.5 mm $CaCl_2$ interface (m); B: DPPC and cholesterol were dissolved in hexane–methanol 95:5 individually and spread successively on the interface (S); concentration of cholesterol was also 3.5 wt%.

Interactions of SP-A with DPPC/cholesterol mixed monolayers

Surface tension–surface area isotherms of DPPC/cholesterol mixed monolayers, formed simultaneously, in the presence of SP-A are depicted in **Fig. 6A**. SP-A was applied either before or after formation of the DPPC/cholesterol mixed monolayer. Results showed that SP-A could not restore the surface activity of DPPC diminished by the presence of cholesterol (as shown in Fig. 4). Very low surface tensions could be attained only with maximum compression at cycle 1, and both γ_{max} and γ_{min} were elevated following the compression/expansion cycles (Table 1). However, SP-A could partially restore the surface activity of DPPC when it was applied on DPPC/cholesterol mixed monolayers formed by successive spreading (Fig. 6B, ●—●). Such films could be compressed to near zero surface tension by reducing the surface area about 40%. When the monolayer was formed by successive spreading of DPPC/SP-A/cholesterol, cholesterol was unable to impair the surface activity of DPPC already interacted with SP-A (Fig. 6B, ○—○). Such monolayers could achieve near zero surface tension with about 25% reduction of the surface area, similar to that observed for pure DPPC monolayers in the presence of SP-A (Fig. 3B). However, both γ_{max} and γ_{min} were increased throughout succes-

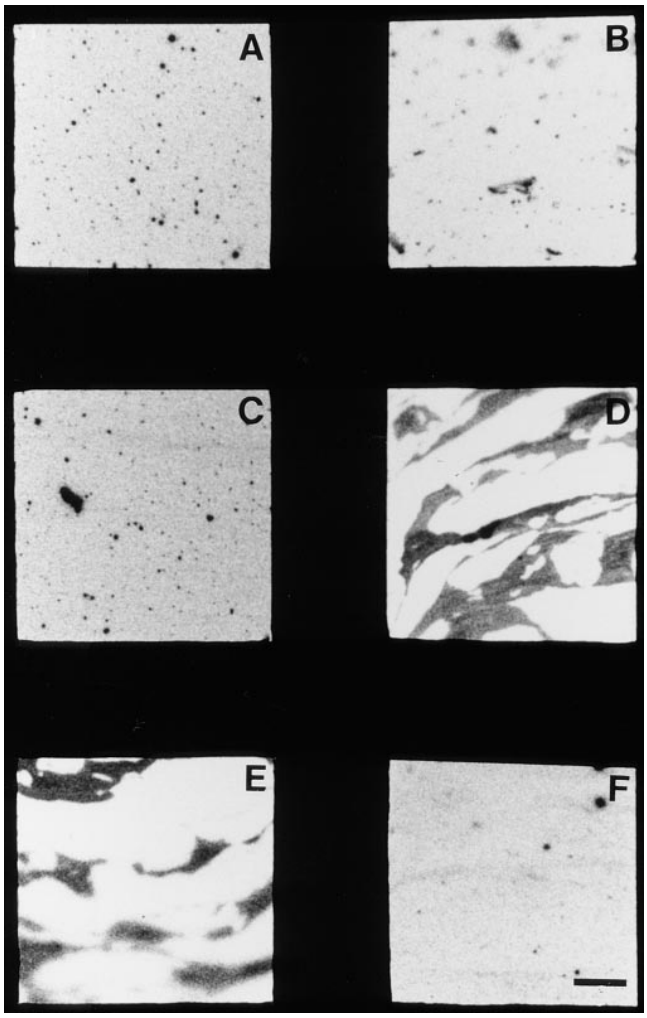

Fig. 5. Langmuir-Blodgett films of mixed DPPC/cholesterol monolayers. All films were deposited in the rhomboid surface balance with constant surface tension of 23–24 mN/m at 37°C except for graph F. A: mixture of [^{14}C]DPPC-labeled DPPC and 3.5 wt% unlabeled cholesterol was spread together on the air/saline, 1.5 mm $CaCl_2$ interface to form a monolayer of 26–27 mN/m and compressed to 23–24 mN/m for film deposition. B: [^{14}C]DPPC-labeled DPPC and 3.5 wt% unlabeled cholesterol was spread successively to reach 26–27 mN/m and compressed to 23–24 mN/m for film deposition. C: experiment as for A except that unlabeled DPPC and [^{14}C]cholesterol were used. D: as for B but unlabeled DPPC and [^{14}C]cholesterol-labeled cholesterol were used. E: unlabeled DPPC was spread to 50 mN/m and 10 wt% [^{14}C]cholesterol-labeled cholesterol was spread successively, the film was then compressed to 23–24 mN/m for deposition. F: experiment was performed as for D except that the experimental temperature was 42.5 ± 0.5°C. The bar indicates 2 mm for all graphs.

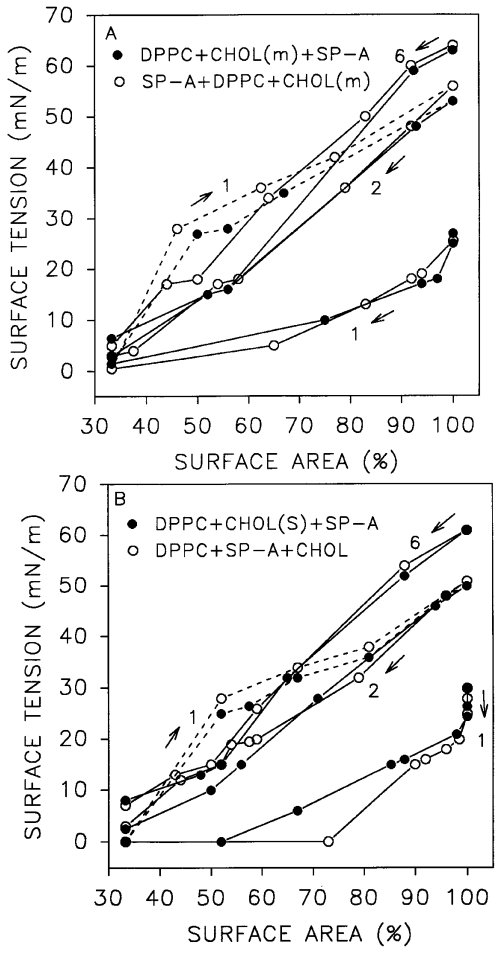

Fig. 6. Representative dynamic compression/expansion cycling of mixed DPPC/cholesterol surface active films in the presence of SP-A at 37°C. Experiments and other details as in Fig. 4. A: DPPC and 3.5 wt% cholesterol were dissolved in the spreading solvent and spread on the interface (m), SP-A was applied before (○—○) or after (●—●) formation of the mixed monolayer; B: DPPC and cholesterol were spread successively (S) in the order of DPPC/cholesterol/SP-A (●—●) or DPPC/SP-A/cholesterol (○—○). In either case, 20 μl of 0.4 mg/ml SP-A in water–2-propanol 2:1 was applied.

sive cycles as in all mixed monolayers containing cholesterol (Table 1).

Figure 7 shows the distributions of DPPC and cholesterol within DPPC/cholesterol mixed monolayers in the presence of SP-A. Figures 7A to 7G were L-B films deposited at the equilibrium surface tension (23–24 mN/m), while 7H was made at 0.5–1.5 mN/m. Figures 7A to 7D pertain to monolayers formed by co-spreading a mixture of DPPC and cholesterol, while 7E to 7H were monolayers formed by successive spreading of DPPC and cholesterol. SP-A was applied before (B) and after (A) the formation of mixed monolayers. Both films (7A and 7B) revealed [^{14}C]DPPC aggregates larger than those observed in Fig. 5A (without SP-A). In addition, the aggregation of [^{14}C]DPPC in Figs. 7A and 7B are similar. These results indicate that SP-A could interact with DPPC even after DPPC had interacted with cholesterol. Figures 7C and 7D pertain to the distribu-

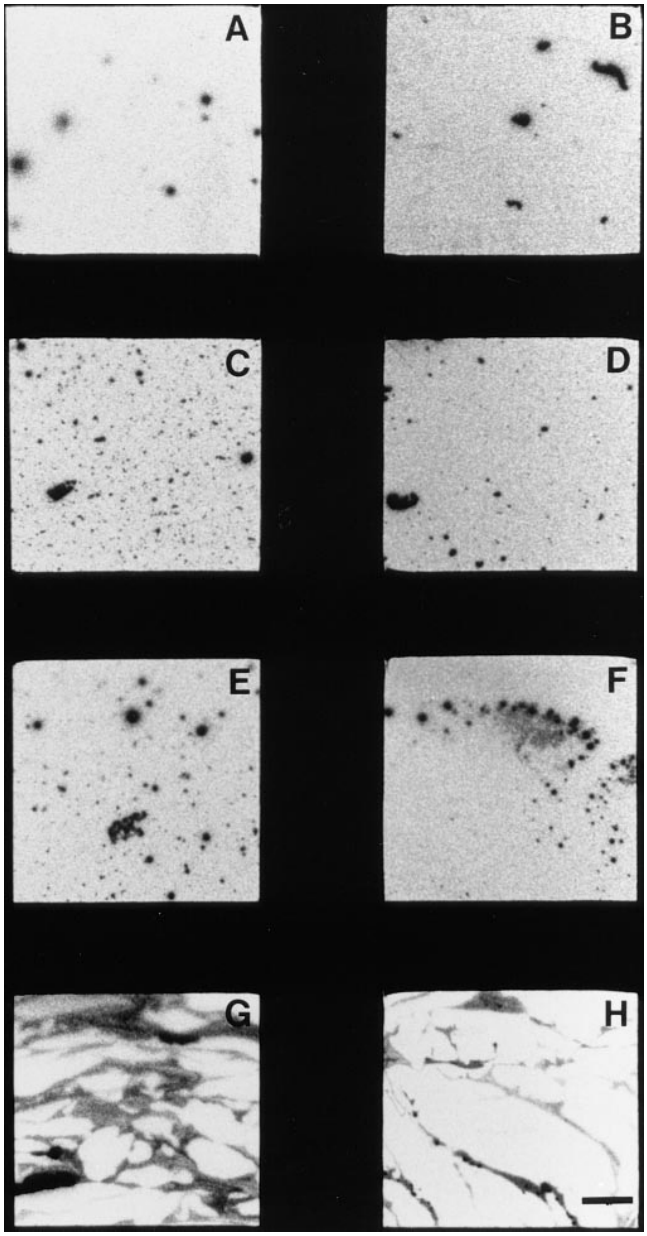

Fig. 7. Langmuir-Blogett films of DPPC/cholesterol mixed monolayers in the presence of SP-A. All films were made at 37°C in the rhomboid surface balance at a constant surface tension of 23–24 mN/m except for graph H which was deposited at a lower surface tension. A: [^{14}C]DPPC-labeled DPPC and 3.5 wt% unlabeled cholesterol dissolved in the spreading solvent were co-spread on the interface to 26–27 mN/m and 20 μl SP-A (0.4 mg/ml in water–2-propanol 2:1) was applied on the mixed monolayer; the monolayer was then compressed to 23–24 mN/m for film deposition; B: as for A except that SP-A was applied on the interface first; C: as for A except that unlabeled DPPC and [^{14}C]cholesterol-labeled cholesterol were used; D: as for B but [^{14}C]cholesterol was used; E: the film was formed by successive spreading of [^{14}C]DPPC-labeled DPPC/cholesterol/SP-A; F: the film was formed by successive spreading of [^{14}C]DPPC-labeled DPPC/SP-A/cholesterol; G: the film was formed by the successive spreading of DPPC/SP-A/[^{14}C]cholesterol; H: as for G except that L-B film was deposited at 0.5–1.5 mN/m. The bar indicates 2 mm for all graphs.

tion of [^{14}C]cholesterol in DPPC/cholesterol mixed monolayers when SP-A was present before (D) and after (C) the formation of mixed monolayers. It appears that SP-A did not affect the organization and aggregation of cholesterol as the L-B films in Figs. 7C and 7D are similar to that in Fig. 5C formed in the absence of SP-A. These results show that SP-A could interact with DPPC but not necessarily with cholesterol in homogeneous mixed monolayers of DPPC and cholesterol. Figures 7E and 7F show autoradiographs of [^{14}C]DPPC where the components were spread successively in the sequence of DPPC/cholesterol/SP-A (E) or DPPC/SP-A/cholesterol (F). Larger and rounder appearing aggregates are found in Fig. 7E than those formed without SP-A in Fig. 5B, indicating that SP-A could cause aggregation of DPPC that had interacted with cholesterol. In Fig. 7F the aggregates appear to be localized, as with pure DPPC plus SP-A (Fig. 2D). These results indicate that the interaction of cholesterol with DPPC in monolayers may be suppressed by the presence of SP-A. These results are consistent with the compression/expansion isotherms for the mixed monolayers (Fig. 6B) which show that cholesterol did not impair the surface activity of DPPC already interacted with SP-A. Figure 7G shows that the organization of [^{14}C]cholesterol in monolayers formed by successive spreading of DPPC/SP-A/cholesterol was similar to that of DPPC/cholesterol without SP-A insofar as separate cholesterol domains were found in both mixed monolayers (compare Fig. 7G with Fig. 5D). In contrast to the mixed monolayers without SP-A (see Fig. 4B and Fig. 5D), mixed monolayers with SP-A were capable of reducing the surface tension to near zero with 25% compression from equilibrium surface area (see Fig. 6B and Fig. 7G), similar to DPPC monolayers in the presence of SP-A (Fig. 3B). These results demonstrate that cholesterol could be readily squeezed out from DPPC/cholesterol mixed monolayers if the DPPC had interacted with SP-A first. This implied that SP-A may limit the lateral association between DPPC and cholesterol domains for the following reasons: *1*) cholesterol appeared to have no effect on the aggregation of DPPC that had interacted with SP-A (compare Fig. 2D with Fig. 7F); *2*) SP-A reduced the surface area reduction required to reach near zero surface tension of DPPC/cholesterol mixed monolayers formed by successive spreading to about 40% (Fig. 4B), while the required surface area reduction was further reduced to 25%, similar to that of pure DPPC monolayers in the presence of SP-A, when DPPC had

interacted with SP-A first (Fig. 4B); *3*) SP-A suppressed the transport of cholesterol to the surface from a bLES(chol) dispersion (19). Although the mixed monolayers formed by the sequential spreading of DPPC/SP-A/cholesterol could reach near zero surface tension as easily as pure DPPC in the presence of SP-A, it is clear that cholesterol could not be squeezed out completely even at very low surface tensions (Fig. 7H). This could account for the decreased stability of films containing cholesterol at low surface tension. The surface tension of such a film typically rises to about 10 mN/m when compression is halted for 5 min after reaching near zero, while DPPC in the presence of SP-A can remain at near zero surface tension without further compression for at least 20 min.

Interaction of SP-A with cholesterol

Pure cholesterol monolayers are relatively incompressible. Cholesterol exhibits a relatively high equilibrium surface tension of 33–34 mN/m. The surface active properties of cholesterol in the presence and absence of SP-A are compiled in **Table 2**. **Figure 8A** shows that at 37°C a pure cholesterol monolayer with an initial surface tension of 33–34 mN/m collapsed near 27 mN/m. Surface tension rose quickly at the onset of expansion and stayed constant to the maximum expansion. Moreover, the surface tension–surface area isotherms remained similar during six dynamic cycles of compression/expansion. Even with a higher initial surface tension of 37–38 mN/m (Table 2, row 2), cholesterol retained the same collapse plateau at 27mN/m, but with a higher γ_{max} after each successive cycle. The collapse surface tension was reduced to 22–23 mN/m when SP-A was applied onto cholesterol monolayers (Table 2, row 3 and Fig. 8B). This lower value remained relatively constant for at least six cycles with increasing γ_{max} at successive cycles. The surface tension did not rise as quickly as with pure cholesterol films at the beginning of the expansion, indicating a smaller loss of cholesterol from the film during overcompression in the presence of SP-A. Similar isotherms were observed when SP-A was applied on the interface prior to the spreading of cholesterol. As the 3β-OH is the sole hydrophilic moiety present in cholesterol, our findings suggest that this group may be responsible for the association with SP-A. As a result, the fluidity of cholesterol was reduced and the film was able to sustain a higher surface pressure. Nonetheless, SP-A did not aggregate cholesterol at the equilibrium surface tension (33–34 mN/m). The autoradiographs of [^{14}C]cholesterol display a homogeneous mist of cholesterol at 33–34 mN/m, similar to that of pure DPPC at 23–24 mN/m (Fig. 2B), both in the presence and absence of SP-A.

DISCUSSION

The goal of this study was to investigate the mechanism by which SP-A facilitates the removal of cholesterol from mixed monolayers of DPPC and cholesterol during compression. A custom-built rhomboid surface balance was used for this study. Data such as the surface area reduction required to reach near zero surface tension for pure DPPC at 37°C are similar to those reported by Notter, Tabak, and Mavis (29) using a modified Wilhelmy surface balance.

Studies by Kuroki and Akino (10) using thin-layer chromatography plates demonstrated that SP-A interacted with PL with the highest affinity toward DPPC. Because SP-A exhibited a strong binding with dipalmitoylglycerol, produced by phospholipase C hydrolysis of DPPC, but failed to bind with lysophosphatidylcholine obtained from the treatment of DPPC with phospholipase A_2 (10), these investigators concluded that SP-A interacted with the non-polar hydrocarbon chains

TABLE 2. Interaction of SP-A and cholesterol at the air/water interface

Monolayers[a]	Initial Surface Tension[b]	Min & Max Surface Tension at[c]					
		Cycle 1		Cycle 2		Cycle 6	
		γ_{min}	γ_{max}	γ_{min}	γ_{max}	γ_{min}	γ_{max}
	mN/m						
1. CHOL	33–34	26.5 ± 0.5	40.0 ± 0.5	27.0 ± 0.7	40.0 ± 1.0	27.0 ± 0.6	41.0 ± 1.5
2. CHOL	37–38	27.0 ± 0.3	42.3 ± 0.9	27.5 ± 0.5	44.3 ± 0.8	27.5 ± 0.8	46.3 ± 0.9
3. CHOL+SP-A	37–38 → 35–36	22.5 ± 0.3	38.8 ± 0.5	22.5 ± 0.5	39.3 ± 0.5	22.5 ± 0.5	42.5 ± 0.5
4. SP-A+CHOL	38–40	22.7 ± 0.4	41.7 ± 0.9	23.0 ± 0.3	44.0 ± 1.2	22.8 ± 0.4	47.7 ± 1.7

Experiments were carried out as in Table 1.

[a]Composition of monolayers (rows 3 and 4) corresponds to the spreading order of each component.

[b]Surface tension of CHOL monolayer fell slightly after the spreading of SP-A (row 3) and 20 μl of SP-A (0.4 mg/ml in water–2-propanol 2:1) was used where SP-A was applied.

[c]Data are mean ± SE for n ⩾ 3. *P* values (two-tailed *t*-test) for the effect of SP-A on the reduction of collapse surface tension of cholesterol are $P_{2,3} < 0.05$ and $P_{2,4} < 0.05$.

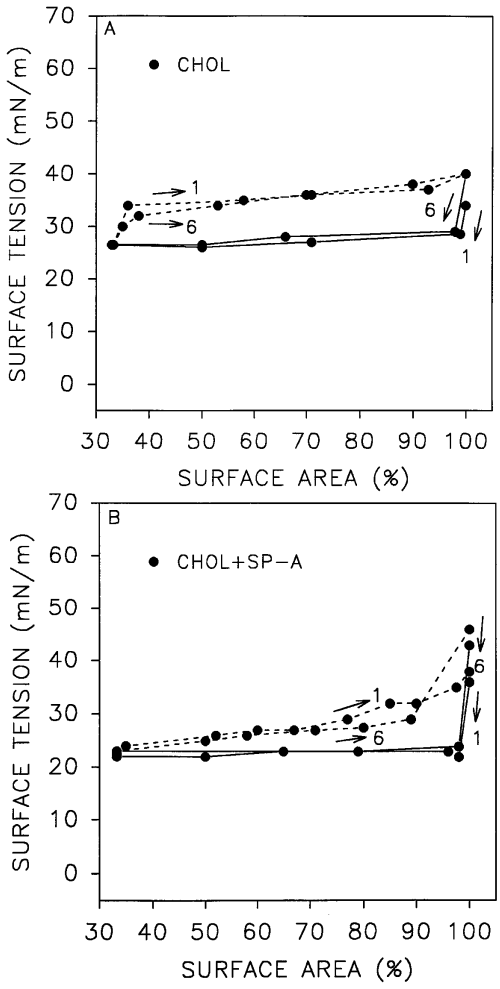

Fig. 8. Representative dynamic compression/expansion cycling of pure cholesterol in the presence and absence of SP-A. Experimental details as in Fig. 3. A: dynamic compression/expansion behavior of cholesterol; B: SP-A was applied on the cholesterol monolayer.

of DPPC. However, the interactions observed here with monolayers seem to be quite different. Our results suggest that SP-A preferentially interacted with the polar head groups of DPPC at the air/water interface. As revealed in Fig. 2C, SP-A aggregated DPPC when SP-A was injected under the monolayer near equilibrium surface tension. DPPC was also aggregated when SP-A in an aqueous medium was applied on DPPC monolayers. In both cases, surface tensions of the monolayers were not affected by the addition of SP-A (Fig. 1A), suggesting that SP-A did not attain or remain at the interface to interact with the non-polar hydrocarbon chains of DPPC. This shows that the interaction of SP-A with DPPC hydrocarbon chains is not required for DPPC monolayer aggregation.

The bulk of our results are consistent with the conclusion that SP-A preferentially interacted with DPPC head groups. Figure 3B shows that SP-A has similar effects on the surface active properties of pure DPPC monolayers, regardless of whether SP-A (dissolved in water–2-propanol 2:1) was applied on the interface before or after formation of the DPPC monolayer. SP-A would be primarily exposed to the polar head groups of DPPC monolayer in the former case and to the hydrocarbon chains in the latter. However, the data in Fig. 1 show that most of the SP-A applied on DPPC monolayers moved through the interface into the subphase. The conclusion that SP-A preferentially interacts with the polar head groups of DPPC monolayers was also supported by other experiments in which SP-A in an aqueous medium (saline, 1.5 mm $CaCl_2$) was applied onto DPPC monolayers (Table 1, row 4). Although application of SP-A in this manner did not alter the surface tension, a similar surface area reduction of about 25% sufficed to achieve the near zero surface tension. Taken together, these studies suggest that the same mechanism is involved in enhancing the surface activity when SP-A in water–2-propanol was applied before or after DPPC monolayer formation as in the case where SP-A in an aqueous medium was applied onto a DPPC monolayer. As the surface tension was not affected in the latter case, we conclude that SP-A must have interacted with DPPC polar head groups in all three cases. A similar interaction may occur with bilayers where the polar head groups are exposed to SP-A, as addition of SP-A to liposomes containing DPPC results in vesicle aggregation (9).

However, our results also show that SP-A may interact moderately with non-polar hydrocarbon chains of DPPC when SP-A in water–2-propanol 2:1 was applied onto a DPPC monolayer (Fig. 1B). When SP-A in such a medium was applied onto a clean air/water interface it quickly diffused into the subphase without altering the surface tension. But the surface tension was reduced slightly when such a SP-A solution was applied onto a DPPC monolayer (30–31 mN/m), indicating that a small portion of SP-A may remain at the interface and associate with the hydrocarbon chains of DPPC. The small reduction (≈2 mN/m) in the surface tension indicates that the majority of SP-A molecules diffused into the subphase below the interface. Some of the SP-A likely interacted with the polar head groups of DPPC monolayer. It should be noted that SP-A could associate with DPPC head groups while still being partially exposed to the air/water interface and reduce the surface tension. Interestingly, a greater surface tension reduction was observed when the same amount of SP-A was applied onto DPPC monolayers at 50 than 30 mN/m (Fig. 1B), suggesting that the ability of SP-A to remain straddled across the surface required unoccupied

surface area. Our studies cannot distinguish which SP-A domain interacts with DPPC, but others have suggested that both the carbohydrate-binding domains (30) as well as the hydrophobic neck region (31) of SP-A are important in phospholipid binding.

Cholesterol can condense DPPC as well as unsaturated PC monolayers (17, 32). This effect has been interpreted as that of cholesterol occupying cavities existing between fatty acyl chains of adjacent DPPC molecules (17). As a result, cholesterol cannot be squeezed out readily from mixed monolayers through repeated compression. The present studies show that cholesterol could not be squeezed out easily from mixed monolayers of DPPC and cholesterol whether the mixed monolayers were formed by spreading the two components simultaneously or successively. Surprisingly, L-B films of the mixed monolayers revealed very different distributions of cholesterol at equilibrium surface tension: a homogeneously mixed phase in the simultaneous case and separate cholesterol domains in the successive case. These results indicate that cholesterol cannot be squeezed out readily from DPPC/cholesterol mixed monolayers whether it is packed between the hydrocarbon chains of DPPC or exists as a separate domain.

With DPPC/cholesterol mixed monolayers formed by successive spreading, cholesterol-rich domains were observed with mixed monolayers containing either 3.5 wt% (Fig. 5D) or 10 wt% (Fig. 5E) cholesterol at 37°C. However, an even distribution of cholesterol appeared when mixed monolayers of DPPC and 3.5 wt% cholesterol were formed at 42.5°C even with successive spreading (Fig. 5F). These results suggest that the distribution of surfactant components in a monolayer is highly dependent on their fluidity. A recent report by Discher et al. (33) also showed that the distribution of pulmonary surfactant components in a monolayer was temperature- and surface pressure-dependent. It has become apparent that cholesterol displays non-ideal miscibility in model bilayer membranes at 37°C (34, 35). Moreover, considerable evidence has accumulated demonstrating that plasma membranes possess cholesterol-rich and cholesterol-poor domains (36–38). These and other observations argue that the classical fluid–mosaic model for biological membranes should be modified to include non-uniform lipid mosaic domains (39, 40). The possibility exists that cholesterol may adopt a non-homogeneous distribution in pulmonary surfactant microstructures, such as lamellar bodies, tubular myelin, and surfactant monolayers. Consequently, the immiscible mixed monolayers formed at 37°C by successive spreading in the present study could reflect lipid distribution in biological systems such as surfactant.

Figure 6A shows that SP-A had no influence on the surface activity of miscible mixed monolayers of DPPC and cholesterol formed by simultaneous spreading; SP-A could not facilitate the removal of cholesterol once it had packed within DPPC hydrocarbon chains despite the observation that SP-A appeared to interact with DPPC (Figs. 7A and 7B). When the mixed monolayer was formed by successive spreading of DPPC/cholesterol/SP-A, SP-A slightly improved (40% area reduction) the surface tension-lowering of this immiscible monolayer during compression. SP-A further facilitated the surface tension-lowering of the immiscible monolayers formed by successive spreading of DPPC/SP-A/cholesterol insofar as the surface area reduction (25%) required to achieve near zero surface tension was similar to that of pure DPPC in the presence of SP-A. With such monolayers (DPPC/SP-A/cholesterol), SP-A not only aggregated DPPC, it also impeded lateral interactions between DPPC and cholesterol domains. Whether SP-A also interacted with cholesterol to improve the surface activity of the immiscible DPPC/cholesterol mixed monolayers is not known, despite the fact that SP-A can interact with pure cholesterol monolayers (Fig. 8). Taken together, these results indicate that in order to squeeze out cholesterol readily from DPPC/cholesterol mixed monolayers, it is critical for DPPC to associate with SP-A first.

In summary, we have investigated the interaction of SP-A with DPPC and cholesterol at the air/water interface with a rhomboid surface balance at 37°C. Our results show that SP-A aggregated DPPC molecules and facilitated the surface tension lowering of DPPC monolayers. Cholesterol existed as separate domains in mixed monolayers formed by successive spreading of DPPC and cholesterol, while homogeneous DPPC/cholesterol mixed monolayers were observed when both components were dissolved together in the spreading medium. Cholesterol could not be squeezed out readily from either mixed monolayer through compression. SP-A reduced the collapse surface tension of pure cholesterol and facilitated the squeeze-out of cholesterol domains from DPPC/cholesterol. The present studies show that successive spreading results in major differences in surface properties and profound alterations in surface morphology. In view of the recent reports on cellular membranes (39, 40), mixed monolayers formed by successive spreading of each component may closely mimic the lipid organizations in biological systems and give new insight into interactions of monolayer components.

This work was supported by MRC Group Grant from the Medical Research Council of Canada (CMRC 268C7). The assistance of Mr. Kevin Inchley with computer skills is gratefully acknowledged.

Manuscript received 3 April 1997, in revised form 1 July 1997, and in revised form 9 September 1997.

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