Adsorption of Pulmonary Surfactant Protein D to Phospholipid Monolayers at the Air−Water Interface†

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ABSTRACT: The intrinsic surface activity of recombinant rat surfactant-associated protein D (SP-D) expressed in CHO-K1 cells has been determined from measurements of surface tension of its aqueous solutions. The interactions of recombinant SP-D with monolayers of phosphatidylcholine (PC), phosphatidylglycerol (PG), and phosphatidylinositol (PI) spread at the air−water interface have been characterized. Injection of SP-D beneath preformed lipid monolayers at surface pressures less than 30 mN/m produced an increase in surface pressure, consistent with SP-D penetrating the lipid films. The adsorption of SP-D to the lipid monolayers did not display significant head group dependency, suggesting that the changes in surface pressure produced by the protein were likely due primarily to hydrophobic interactions with the lipid layers. In the presence of calcium ions in the subphase, SP-D displayed lower surface activity by itself and a reduced ability to generate surface pressure changes during adsorption to lipid monolayers compared to these properties of the protein in the absence of 2 mM Ca^{2+}. Circular dichroism measurements on SP-D solutions with or without Ca^{2+} suggested that the cations altered the conformation of the protein and this possibly led to the calcium dependency of the surface activity of the protein in the presence or absence of lipid monolayers. Compressional isotherms of surface pressure versus area for SP-D/(DPPC−PI) and SP-D/(DPPC−PG) films formed by adsorption of the protein to preformed lipid monolayers were consistent with incorporation of some or all of the SP-D molecules into the lipid layers. The isotherms obtained on compression of the SP-D/lipid films to a maximum surface pressure of about 70 mN/m were consistent with the interpretation that any SP-D which was incorporated by adsorption was apparently not squeezed out, nor was lipid removed by the protein. The work suggests that when soluble recombinant SP-D is allowed to interact with phospholipid monolayers under the selected conditions of this experiment, it does so to a limited extent driven by primarily hydrophobic forces, and apparently without a high selectivity for phospholipid head groups.

SP-D[1] is associated with pulmonary surfactant through carbohydrate- and calcium-dependent interactions (Persson et al., 1990). In vitro measurements have shown that SP-D binds to glycolipids, among them the phosphoglycolipid PI. Experiments with surfactant-associated phospholipids in the solid state [on thin-layer chromatography plates (Ogasawara et al., 1992; Persson et al., 1992) or coated on microtiter wells (Ogasawara et al., 1992)] or incorporated into liposomes (Persson et al., 1992) have demonstrated strong specificity of SP-D for PI, with the interaction of SP-D with PI being dependent on calcium and inhibited by competing saccharides (Persson et al., 1992). Both the sugar binding specificity of SP-D (Ogasawara & Voelker, 1995a) and interactions with the hydrocarbon chains of the glycolipids (Ogasawara et al., 1992; Ogasawara & Voelker, 1995a) are likely responsible for the binding of SP-D to glycolipids.

The relevance of carbohydrate- and lipid-binding activity of SP-D for its functions in lung surfactant is not well understood. SP-D might play a role in pulmonary host defence (Crouch et al., 1994) and it might contribute through specific lipid−protein interactions to the extracellular reorganization of pulmonary surfactant (Persson et al., 1992; Kuroki et al., 1991; Ogasawara et al., 1992).

SP-D is a hydrophilic glycoprotein composed of 43-kDa polypeptides characterized by four distinct domains: a noncollagenous NH_2-terminal region involved in intermolecular disulfide bonding, a collagenous domain, a short α-helical sequence, and a noncollagenous carbohydrate recognition C-terminal domain (Shimizu et al., 1992; Crouch et al., 1993). In solution, SP-D is assembled as a homopolymer of four identical trimeric subunits, yielding dodecamers that can further associate to form higher order oligomers (Crouch et al., 1994). The theoretical isoelectric point of rat SP-D is 7.65 (Shimizu et al., 1992), which is consistent with the pI of 6−8 determined by two-dimensional isoelectric focusing/SDS electrophoresis (Persson et al., 1989).

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[1] Abbreviations: CD, circular dichroism; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; EDTA, ethylenediaminetetraacetic acid; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; PG, 1-(3-*sn*-phosphatidyl)-*rac*-glycerol; PI, 1-(3-*sn*-phosphatidyl)-D-*myo*-inositol; SP-A, pulmonary surfactant protein A; SP-D, pulmonary surfactant protein D.

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In this study we employed surface balance techniques to study the interactions of recombinant rat SP-D with monomolecular layers of surfactant-associated phospholipids, PC, PG, and PI, at the air−water interface. The specificity of the lipid polar head groups for insertion of SP-D into phospholipid monolayers and the effect of calcium on this process were investigated. We also examined a potential role for SP-D in the process of selective squeeze-out of non-DPPC components, especially PI, during compression of mixed phospholipid monolayers.

EXPERIMENTAL PROCEDURES

Materials. The recombinant protein was prepared from culture media of CHO-K1 cells transfected with PEE14 plasmid vector harboring the cDNA for rat SP-D (Ogasawara & Voelker, 1995b). The protein was purified by affinity chromatography on mannose-Sepharose (Fornstedt & Porath, 1975; Ogasawara & Voelker, 1995b). This purification procedure yields homogeneous preparations of recombinant SP-D that have essentially identical properties to the protein prepared from lung lavage (Ogasawara & Voelker, 1995a). The recombinant protein interacts with phosphatidylinositol and glucosylceramide in a manner that is indistinguishable from the native protein. Analysis by gel filtration also demonstrates that the recombinant protein forms the same types of oligomers as the native protein. Protein concentration was determined by the BCA assay (Pierce) using bovine serum albumin as the standard. Recombinant rat SP-D was dissolved in 0.15 M NaCl, 5 mM Tris, and 0.5 mM EDTA (pH 7.4). SP-A was isolated from porcine lung lavage as described previously (Taneva et al., 1995). PI (sodium salt) from bovine liver was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). PC and PG (sodium salts) from egg yolk and DPPC were obtained from Sigma Chemical Co. (St. Louis, MO). The lipids were found to be pure by thin-layer chromatography. Each phospholipid showed a single spot on thin-layer chromatography on silica gel with a solvent system of chloroform−methanol−water (65:25:4 by volume). Sodium chloride and calcium chloride from Fisher Scientific Co. (Ottawa, ON) and tris(hydroxymethyl)aminomethane hydrochloride from Sigma Chemical Co. (St. Louis, MO) were reagent grade. Water was deionized and doubly distilled in glass, the second distillation being from a dilute potassium permanganate solution.

Monolayer Measurements. Adsorption measurements were performed at constant surface area in a Teflon dish (R = 1.2 cm) with a subphase volume of 5 mL. Lipid monolayers were spread at the air−water interface from chloroform to give an initial surface pressure, π_i. Fifteen minutes after monolayer formation, desired volumes of SP-D in 0.15 M NaCl, 5 mM Tris, and 0.5 mM EDTA were injected below the monolayer-covered surface through an injection septum. Surface tension was measured as a function of time by the Wilhelmy plate method, using a roughened platinum plate (perimeter of 2 cm) and a computer controlled transducer readout, TSAR 1 (Tech-Ser Inc., CA), connected to a chart recorder (Servoscribe). The subphase (0.15 M NaCl and 5 mM Tris, pH 7.4) was stirred continuously with a Teflon-coated stirring bar and a magnetic stirrer. In similar experiments we examined the effect of 2 mM $CaCl_2$ in the subphase buffer on the adsorption characteristics of SP-D in the presence or absence of preformed phospholipid monolayers.

Measurements of compressional surface pressure-area isotherms for SP-D/lipid films were carried out by using a Teflon Langmuir trough (12 × 5 × 0.5 cm). To minimize possible film leakage, the trough and the Teflon barrier were "primed" before use following the procedure of Hildebran et al. (1979). SP-D, at a concentration of 2.45 μg/mL, was dissolved in the subphase buffer (0.15 M NaCl, and 5 mM Tris, pH 7.4) and within 5 min the phospholipid monolayer was spread from chloroform on the protein surface. It is worth noting that, at this subphase concentration, the adsorption of SP-D did not lead to detectable changes in surface pressure of the clean air−water interface in the first 10 min (Figure 1, curve 3). Twenty minutes was allowed for adsorption of the protein and equilibration of the protein/phospholipid films. The area of the lipid/protein films was reduced manually in a stepwise fashion and surface pressure was monitored after each compression step until equilibrium was reached. The total compression took about 2.5 h. The Wilhelmy plate method (perimeter of the platinum plate was 5 cm) and the strain gauge described above were used to measure the surface pressure. Results from the measurements were presented as surface pressure, π, versus molecular area per phospholipid molecule, F'. All experiments were performed at 22−24 °C.

Circular Dichroism Spectroscopy. CD spectra were recorded at 22 °C on a Jasco Model J-500A spectropolarimeter using 0.05 cm path length cells. SP-D concentration in either 0.15 M NaCl, 5 mM Tris, and 0.5 mM EDTA (pH 7.4) or 0.15 M NaCl, 5 mM Tris, 0.5 mM EDTA, and 5 mM $CaCl_2$ (pH 7.4) was 0.35 mg/mL. The CD spectrum of porcine SP-A in 5 mM HEPES (pH 7.4), at a concentration of 0.2 mg/mL as determined by the fluorescamine method (Udenfriend et al., 1972), was also measured. The data were obtained by accumulation of 32 scans from 260 to 190 nm, each run at a scan speed of 50 nm/min. An average molecular mass of 110 Da was used for calculations of the elipticity per protein residue, Θ.

RESULTS

Adsorption of SP-D to Phospholipid Monolayers Preformed at the Air−Water Interface. Surface pressure, π, defined as $\pi = \sigma_0 - \sigma$, where σ_0 is the surface tension of the clean air−water interface and σ is the surface tension of the protein solution, was measured as a function of time for various subphase concentrations (C_s) of SP-D in the absence of preformed lipid films. When the subphase concentration was less than 1 μg/mL, no changes in π for the protein solutions were detected up to 70 min. The effects of higher concentrations of SP-D in the subphase on surface pressure of the buffer solution are shown in Figure 1.

The interactions of SP-D in solution with spread monolayers of phospholipids were primarily studied using a subphase concentration of SP-D, C_s = 0.7 μg/mL, where the protein by itself displayed negligible surface activity. Figure 2 shows a typical recording of surface pressure as a function of time for monolayers of PI spread at initial surface pressure π_i = 10 mN/m, prior to and after the injection of SP-D into the subphase to a final concentration of 0.7 μg/mL. The adsorption of the protein to the phospholipid monolayer produced a change in the surface pressure, $\Delta\pi$, of about 5 mN/m. In Figure 3, the changes in surface pressure, $\Delta\pi$, following the injection of SP-D beneath the monolayers of PC, PG, and PI preformed at initial surface

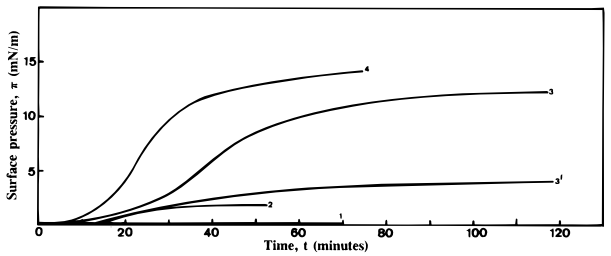

FIGURE 1: Typical curves for time dependence of surface pressure during adsorption of SP-D from aqueous solutions at concentrations C_s (in micrograms per milliliter) of 0.70 (1), 1.05 (2), 2.45 (3, 3′), and 3.50 (4). The subphase was 0.15 M NaCl and 5 mM Tris, pH 7.4 (curves 1−4) or 0.15 M NaCl, 5 mM Tris, and 2 mM $CaCl_2$, pH 7.4 (curve 3′). Duplicates were run for curves 3 and 3′, and the reproducibility of the values was 0.5−1 mN/m along the full length of the isotherms.

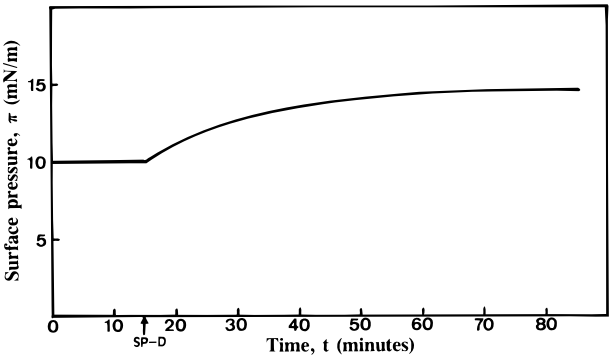

FIGURE 2: Surface pressure as a function of time for monolayers of PI spread to an initial surface pressure of 10 mN/m on a subphase of 0.15 M NaCl and 5 mM Tris, pH 7.4. At the indicated point, SP-D was injected into the subphase to a final concentration of 0.70 μg/mL. Duplicates for PI monolayers were superimposable.

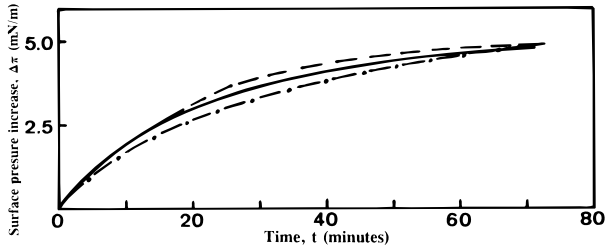

FIGURE 3: Representative curve of surface pressure increase after injection of SP-D underneath a monolayer of PC (- - -), PG (-·-), or PI (—). The initial pressure of the lipid monolayer was 10 mN/m. The protein concentration was 0.70 μg/mL in a subphase of 0.15 M NaCl and 5 mM Tris, pH 7.4. Duplicates for each phospholipid were run; the range of values for the average curves did not exceed 1 mN/m.

pressure $\pi_i = 10$ mN/m were plotted as a function of time. SP-D produced an equivalent increase in surface pressure for the three lipid monolayers. Since this particular protein concentration, $C_s = 0.7$ μg/mL, caused no change in surface tension when the protein was injected into the subphase in the absence of preformed phospholipid monolayer (curve 1, Figure 1), the increase in surface pressure seen in the presence of the lipid films suggested that the protein was likely incorporated into the films. The interpretation where SP-D was in sufficiently close contact to the lipid head groups to induce a change in packing density without the protein being inserted in the monolayer cannot be fully discounted by the current observations but seems unlikely in the light of the lack of effect of the protein on monolayers spread at 30 mN/m (see below). In Figure 4, $\Delta\pi$ versus time curves for adsorption of SP-D at two different subphase

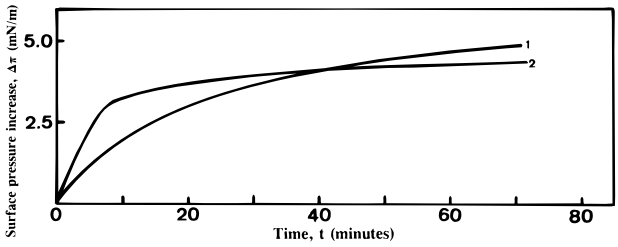

FIGURE 4: Representative curve of surface pressure increase after injection of SP-D underneath a monolayer of PI preformed at initial surface pressure $\pi_i = 10$ mN/m. The protein concentration was 0.70 μg/mL (1) and 2.45 μg/mL (2) in a subphase of 0.15 M NaCl and 5 mM Tris, pH 7.4. The range of values for the average curve of two experiments did not exceed 1 mN/m.

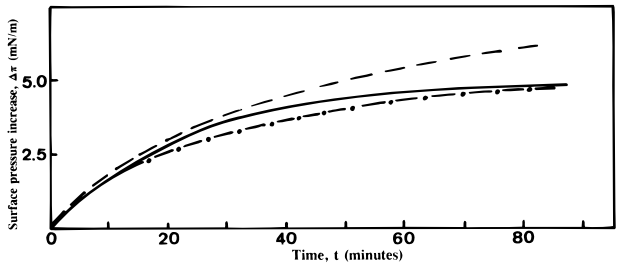

FIGURE 5: Representative curves of surface pressure increase after injection of SP-D beneath a monolayer of PC (- - -), PG (-·-), and PI (—) spread at $\pi_i = 3.5$ mN/m. The concentration of SP-D was 0.70 μg/mL in 0.15 M NaCl and 5 mM Tris, pH 7.4. Duplicates for each of the lipids were run; the range of values of the average did not exceed 1 mN/m.

concentrations into preformed films of PI at $\pi_i = 10$ mN/m demonstrate that a 3.5-fold increase in the protein concentration in the subphase increased the rate of adsorption (the initial adsorption rate was increased about 3-fold and the time required to obtain half of the maximum $\Delta\pi$ was decreased by approximately a factor of 2 by the 3.5-fold increase in concentration) but it did not affect the final value of the surface pressure change produced by the protein. It is worth noting that adsorption of proteins is a slow process where times on the order of hours are required to enable accurate measurements of equilibrium surface pressures. At $C_s = 2.45$ μg/mL the adsorption of SP-D to the clean air−water interface gave rise to $\pi \approx 12$ mN/m (curve 3 in Figure 1), whereas in the presence of the phospholipid monolayers $\Delta\pi$ was about 5 mN/m (curve 2 in Figure 4). Therefore, at this protein concentration the lipid films decreased $\Delta\pi$ produced by SP-D below the value measured for the adsorption of the protein to the clean air−water interface.

Measurements of $\Delta\pi$ versus time were made when the initial surface pressure of the spread phospholipid monolayer, π_i, varied and the concentration of SP-D in the subphase, C_s, was held constant. Comparison of results for $\pi_i = 3.5$ mN/m (Figure 5) with those for $\pi_i = 10$ mN/m (Figure 3) at $C_s = 0.7$ μg/mL suggested that, at least at relatively low surface pressures, the increase in the surface pressure $\Delta\pi$ due to adsorption of SP-D to the phospholipid monolayers was independent of the initial surface pressure of the preformed lipid film. The similarity of the $\Delta\pi$ vs time profiles for the three phospholipids in Figure 5 confirmed the observation that effectively SP-D did not preferentially interact with either of the phospholipids in this process of protein adsorption into lipid films at the air−water interface. When SP-D ($C_s = 0.7$ or 2.45 μg/mL) was injected below monolayers of PI, PG, or PC preformed at π_i of about 30

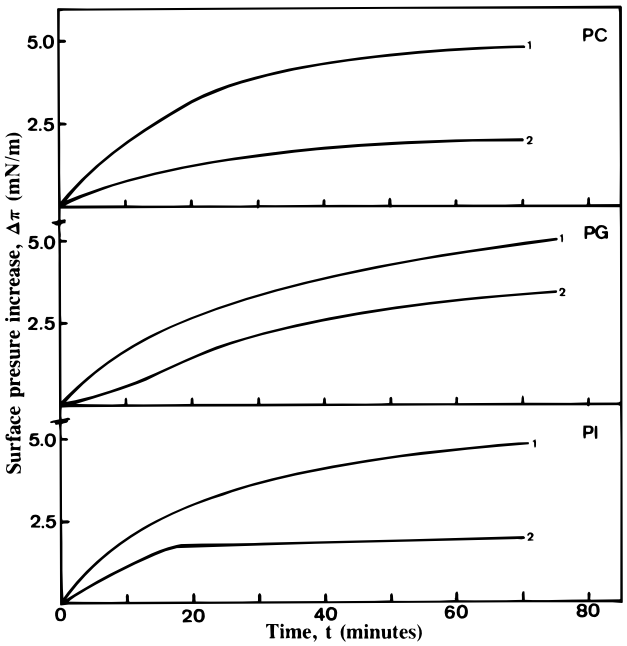

FIGURE 6: Effect of calcium ions on the adsorption of SP-D to phospholipid monolayers of PC, PG, and PI. SP-D was injected beneath lipid films that had been spread at $\pi_i = 10$ mN/m. The protein concentration was 0.70 μg/mL in a subphase of 0.15 M NaCl and 5 mM Tris, pH 7.4 (curves 1) or 0.15 M NaCl, 5 mM Tris, and 2 mM $CaCl_2$, pH 7.4 (curves 2). The curves shown are representative of two measurements for each experiment. The range of values for the average did not exceed 1 mN/m.

mN/m, the protein was no longer able to cause an increase in surface pressure (data not shown).

Effect of Calcium Ions on Interactions of SP-D in the Subphase with Spread Phospholipid Monolayers. The influence of Ca^{2+} on the adsorption of SP-D to preformed monolayers of PC, PG, and PI was studied in the presence of 2 mM $CaCl_2$ in the subphase (0.15 M NaCl and 5 mM Tris, pH 7.4). Figure 6 compares the changes in the surface pressure $\Delta\pi$ during adsorption of SP-D to the lipid films in the absence (curves 1) and presence (curves 2) of Ca^{2+} in the subphase. For the three phospholipids calcium appeared to reduce $\Delta\pi$ caused by the protein adsorption in comparison to the values measured in the absence of Ca^{2+}. A similar effect of Ca^{2+} was observed when SP-D was injected beneath monolayers of PC, PG, and PI spread at $\pi_i = 3.5$ mN/m (data not shown).

In separate experiments, effects of Ca^{2+} on the properties of monolayers of PC, PG, and PI were examined by injecting Ca^{2+} to a final concentration of 2 mM into the subphase after the lipid films had been preformed at $\pi_i = 10$ mN/m (data not shown). In these experiments Ca^{2+} did not affect the constancy of π_i and this implied that the divalent ions at this concentration in the subphase did not alter the lateral packing density of the phospholipids. Likewise, 2 mM Ca^{2+} in the subphase did not significantly modify the surface pressure−area isotherms for each of the phospholipids measured by continuous compression of the spread monolayers (data not shown). This suggests that the intrinsic expanded nature of these unsaturated films was not transformed by the level of calcium used, which is in contrast to the condensing effect of calcium on films of saturated negatively charged lipids such as dipalmitoylphosphatidylglycerol (e.g., Nag et al., 1994).

Measurements of surface pressure during adsorption of SP-D to the clean air−water interface in the presence of 2 mM Ca^{2+} in the subphase showed that the divalent ions attenuated the intrinsic surface activity of the protein (compare curves 3 and 3′ in Figure 1). To examine potential modifications of the secondary structure of SP-D induced by Ca^{2+}, CD spectra of SP-D in the absence or presence of 5 mM $CaCl_2$ were measured (curves 1 and 2 in Figure 7). Compared to the CD spectrum of SP-A (curve 3 in Figure 7), the spectra for SP-D were of lower intensity and their minima were shifted to lower wavelengths. The spectra for SP-D were ambiguous in that they were not representative of any classical well-characterized secondary structure. With a negative band at about 200 nm, the spectra for SP-D were similar to those observed for proteins in random coil or unordered conformation (Johnson, 1988; Perczel et al., 1991). However, the positive CD intensity at about 220 nm, typical for proteins in random coil (Johnson, 1988), was replaced by a negative shoulder in the spectrum of SP-D. The spectra obtained were not used to compute the proportion of secondary structure present by the use of the usual algorithms, but previous analysis of this type of spectra has shown very little α-helix, no parallel β-sheet, and a fair amount of antiparallel β-sheet and β-turn (Johnson, 1988). The long arms of the SP-D may lead to a variety of spatial orientations of the dodecomeric and multimeric SP-D structures (Crouch et al., 1994) and their asymmetry at the level of quaternary structure could be contributing to the observed spectra. Calcium ions mostly affected the intensity of the extremum at 200 nm and this decrease in the negative elipticity of SP-D suggested that the cations possibly caused some changes in the secondary structure of the protein.

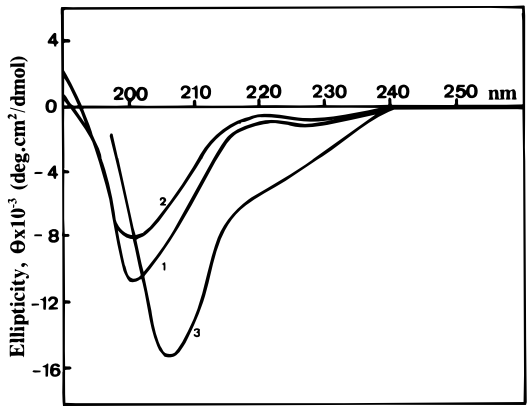

FIGURE 7: Circular dichroic spectra of SP-D in 0.15 M NaCl, 5 mM Tris, and 0.5 mM EDTA, pH 7.4 (1), or 0.15 M NaCl, 5 mM Tris, 0.5 mM EDTA, and 5 mM $CaCl_2$, pH 7.4 (2), and SP-A in 5 mM HEPES, pH 7.4 (3).

Compressional Surface Pressure−Area Isotherms for the SP-D/Lipid Films. Figure 8 shows isotherms of surface pressure versus mean area per phospholipid molecule, F', for monolayers of DPPC−PI (7:3 mol/mol) spread on protein-free subphases (curve 1) and on subphases that contained 2.45 μg/mL of either SP-D (curve 2) or SP-A (curve 3). At the start of compression each of the films of DPPC−PI contained an equal amount of lipid corresponding to a monolayer area of 1.2 nm^2/lipid molecule. We were unable to tell the amounts of the proteins incorporated into the surface films from the subphase. The $\pi(F^0)$ isotherm for the DPPC−PI monolayers spread on protein-free subphases (curve 1, Figure 8) displayed a kink point at about 35 mN/m, which has been associated with a squeeze-out of a phase enriched in the unsaturated lipid component, i.e., PI (Boonman et al., 1987; Egberts et al., 1989; Pastrana-Rios

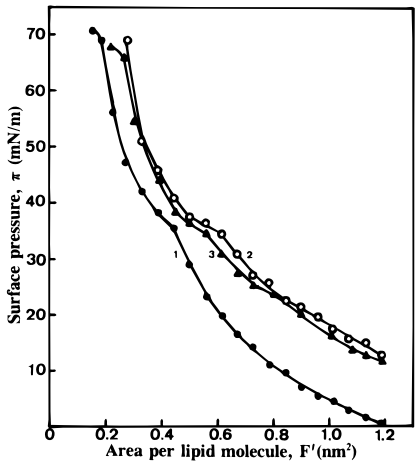

FIGURE 8: Representative isotherms of surface pressure versus area per phospholipid molecule for monolayers of DPPC−PI (7:3 mol/mol) spread on subphases of 0.15 M NaCl and 5 mM Tris, pH 7.4, containing no protein (1), SP-D (2), or SP-A (3). The concentration of the protein in the subphase was 2.45 μg/mL. Duplicates were run for each curve and the reproducibility of the values was $\pm$0.02 nm^2/molecule.

et al., 1994). To check which component was squeezed out at the low pressures during compression of the DPPC−PI films, we compared the compressional isotherm for the films, recalculated on an area per DPPC molecule scale, with the isotherm for the monolayers of DPPC alone, measured under similar experimental conditions (data not shown). At $\pi \leq$ 45 mN/m the curve for the DPPC−PI films was displaced to higher areas than the isotherm for DPPC alone, consistent with some PI being present in the binary monolayers in the range of pressures between 0 and 45 mN/m. At $\pi >$ 45 mN/m the two curves overlapped, within the accuracy of determination of the monolayer areas ($\pm$ 0.02 nm^2/molecule), and this was consistent with preferential squeeze-out of PI from the DPPC−PI monolayers and formation of an almost pure DPPC film at $\pi >$ 45 mN/m. Similar experimental observations have already been reported for DPPC−PI monolayers (Egberts et al., 1989).

Inflection points at $\pi \approx$ 35 mN/m were also seen in the $\pi(F')$ plots for the monolayers of DPPC−PI formed on subphases which contained either SP-D or SP-A (curves 2 and 3 in Figure 8). A collapse pressure of about 70 mN/m was measured for the DPPC−PI monolayers that was independent of the compositions of the subphases. The π-(F') isotherms measured for the films of DPPC−PI spread on the surfaces of the protein solutions (curves 2 and 3, Figure 8) were shifted to the higher areas compared to the isotherm for the lipid monolayer in the absence of protein (curve 1, Figure 8). This observation was consistent with some SP-D or SP-A being present in the phospholipid monolayers in the whole range of surface pressures.

Compressional surface pressure-area isotherms were measured for films of DPPC−PG (7:3 mol/mol) spread on either buffer or solutions containing SP-A or SP-D (C_s = 2.45 μg/mL) (data not shown). Kink points at about 35 mN/m and collapse pressures of about 70 mN/m were characteristic for the isotherms. In the whole range of surface pressures measured, the areas per phospholipid molecule in the presence of protein (F'), were larger than the areas per lipid molecule in the absence of protein, F^0. This suggested that SP-D and SP-A were likely incorporated into the lipid monolayers and they were at least partly present in the surface films that had been compressed to $\pi \approx$ 70 mN/m.

DISCUSSION

Adsorption of SP-D to the Air−Water Interface. Recombinant rat SP-D exhibited an intrinsic tendency to adsorb to the air−water interface. SP-D showed surface activity similar to that of SP-A, e.g., at subphase concentration C_s = 2.45 μg/mL, an equilibrium surface pressure of about 12 mN/m was measured for SP-D (Figure 1), comparable to 14 mN/m observed for SP-A at C_s = 2 μg/mL (Hawgood et al., 1985). Compared to other water-soluble proteins, e.g., lysozyme or β-casein, SP-D displayed a lower ability to accumulate at the air−water interface. Lysozyme at C_s = 0.56 μg/mL gave rise to $\pi \approx$ 9 mN/m and β-casein at C_s = 1 μg/mL produced $\pi \approx$ 19 mN/m (Phillips et al., 1975), whereas at similar subphase concentrations SP-D did not show appreciable change in surface pressure.

Interactions of SP-D with Phospholipid Monolayers. The presence of lipid monolayers at the surface enhanced the attraction of SP-D to the interface since such monolayers were modified in the presence of concentrations of SP-D that otherwise did not enter a clean interface to any significant extent. The interactions between a protein adsorbing to a lipid-covered surface and the lipid can depend on a number of factors: (i) nature of the lipid polar head groups and the arrangements and charges of these moieties; (ii) the length, extent of saturation, and lateral packing density of the hydrocarbon chains; (iii) intrinsic surface activity of the protein; (iv) presence of ions in the subphase; (v) accessibility of interacting groups in lipid and protein to one another; and (vi) the strength of the interaction between the protein and the lipid (Demel et al., 1973; Ahlers et al., 1991). These interactions may lead to insertion of proteins into lipid films, accompanied by a simultaneous increase in the surface pressure, $\Delta\pi$, of the monolayer. On the other hand, a protein may not insert into the lipid phase but nevertheless a lipid monolayer might cause accumulation and orientation of protein molecules at the surface; e.g., polypeptides interacting solely with the lipid head groups through electrostatic interactions without penetration into the monolayer have been shown not to affect the surface pressure (Demel et al., 1973).

After injection of SP-D beneath the preformed monolayers of each of the phospholipids used in the present study, an increase in surface pressure, $\Delta\pi$, was observed, suggesting that at least part of the protein was incorporated into the lipid monolayers (Figures 3 and 5). At low protein concentrations, e.g., C_s = 0.7 μg/mL, $\Delta\pi$ produced by SP-D was zero at the clean air−water interface but it was positive when lipid monolayers were present (compare curve 1, Figure 1, with Figures 3 and 5). This observation can be interpreted as resulting from an enhanced protein adsorption arising from lipid−protein interactions. The $\Delta\pi(t)$ results for adsorption of SP-D into phospholipid monolayers in Figures 3−5 indicate that the final value of $\Delta\pi$ was about 5 mN/m and it was independent of both the initial surface pressure of the preformed lipid monolayers, π_i (Figures 3 and 5), and the subphase concentration of the protein (Figure 4). Thus, $\Delta\pi \approx$ 5 mN/m appeared to be a limit of the "penetration power" of SP-D into the three phospholipids studied. At sufficiently high initial surface pressures, e.g., $\pi_i \approx$ 30 mN/m, the adsorption of SP-D to the films of PC, PG, and PI did not lead to increase in surface pressure. A value of $\pi_i \approx$ 30 mN/m has been determined as a limit for other soluble proteins to penetrate into phospholipid-covered surfaces (Phillips et al., 1975; Weinberg et al., 1992).

Analysis of $\Delta\pi(t)$ curves for adsorption of SP-D to the monolayers of PC, PG, and PI (Figures 3 and 5) revealed that the values for $\Delta\pi$ were similar for the three phospholipids and they were independent of the natures and charges of the lipid head groups. PC is zwitterionic and forms essentially neutral monolayers over a large range of pH values (Phillips & Chapman, 1968); PG forms fully deprotonated monolayers at pH > 4 for subphase ion concentrations > 10 mM (Lakhdar-Ghazal et al., 1983). PI carries a single negative charge (Gunstone et al., 1994) and it presumably forms negatively charged monolayers at neutral pH (Reig et al., 1988). Since pH 7.4 used in the penetration experiments in this study was close to the isoelectric point of SP-D (Persson et al., 1989), the protein possibly had a zero net charge and likely it did not interact with the lipid monolayers through electrostatic forces. This assumption was strengthened by the similarity in the $\Delta\pi(t)$ profiles for adsorption of SP-D to neutral (PC) and negatively charged (PG and PI) phospholipids (Figures 3 and 5).

The three phospholipids had similar acyl chain distributions, e.g., the ratios of saturated to unsaturated acyl chains were 0.91 for PI, according to the supplier's catalogue, and 0.93 for PC and PG, determined by gas chromatography in this laboratory. The observation that at pH 7.4 SP-D had similar affinities, independent of the head groups of the lipids, to insert into monolayers of PC, PG, and PI, which had comparable compositions of their hydrocarbon chains, suggested that possibly hydrophobic forces played a significant role in the interactions of SP-D with the lipid films at this pH.

The head group independency of the interactions of SP-D with the monolayers of PC, PG, and PI observed in the present study is at variance with the strong specificity of SP-D for PI seen in experiments with the phospholipid coated on thin-layer chromatography plates and microtiter wells (Ogasawara et al., 1992; Person et al., 1992) or incorporated into DPPC liposomes (Persson et al., 1992). In this study the preformed monolayers of PI represented a surface with a potential specific functional group, i.e., inositol moiety, for interaction with SP-D in the subphase solution. Changes in surface pressure $\Delta\pi$ were taken as measures for the ability of the protein to interact with and insert into the lipid monolayers. From measurements of $\Delta\pi$ alone, the actual amounts of SP-D adsorbed to the lipid films cannot be determined. If SP-D in the subphase interacted specifically with the head groups of PI, it is likely that an enhanced accumulation of SP-D beneath this particular lipid occurred compared to the amounts adsorbed during nonspecific adsorption of the protein to PC and PG films. However, if there was a limit of penetration for SP-D into the region of the hydrocarbon lipid chains, this limit being governed by the intrinsic surface activity of SP-D, than an apparent head group independency of $\Delta\pi$ induced by the protein might be observed.

Apart from the fact that measurements of $\Delta\pi$ alone were likely insensitive to the actual amounts of SP-D present at the hydrophilic surfaces of the lipid monolayers, we should consider other aspects of the physical model used in the present study to evaluate lipid−protein interactions. Specific interactions between proteins in the subphase and spread lipid monolayers containing specific functional groups have been demonstrated, and they have been shown to be efficient when the interacting components have suitable spatial orientation and compatibility (Haas & Möhwald, 1989; Ahlers et al., 1991). A simple increase in spacing in charged lipid head groups as would be expected in DPPC−PI and DPPC−PG monolayers did not suggest that packing density of the head groups had an influence on the apparent lack of specificity in the interaction. It also seems important that SP-D adsorbing to the lipid monolayer should be suitably oriented with respect to the lipid layer, so that potential binding site(s) for inositol are aligned toward the lipid head groups rather than away toward the aqueous phase. Another factor that might modify the ability of SP-D to interact with certain functional groups is a potential change in the conformation of the protein caused by the adsorption of SP-D to the lipid-covered surface. For some water-soluble proteins, interactions with phospholipid membranes (vesicles) have been accompanied by conformational changes in the protein (Maget-Dana & Ptak, 1995).

Effect of Ca^{2+} on the Interactions of SP-D with Lipid Monolayers. Calcium ions are required for interactions of SP-D with PI and specific saccharides in vitro, and they play a role in the carbohydrate-dependent association of SP-D with pulmonary surfactant (Persson et al., 1990, 1992; Ogasawara et al., 1992). The exact mechanism by which Ca^{2+} mediates binding of SP-D to PI and carbohydrates is not known. In the present study, the ability of SP-D to adsorb and penetrate into the lipid monolayers appeared to be sensitive to the presence of Ca^{2+} in the subphase (Figure 6). Since our results indicated an absence of effect of 2 mM $CaCl_2$ on the monolayer characteristics of PC (neutral) or PG and PI (negatively charged), it seems that the cations hardly modified the lipid−protein interactions through changes in the charges of the lipid polar head groups or in the intermolecular packing of the phospholipids. It is noted, however, that 5 mM calcium did cause an increase in order of surfactant lipids in bilayers (Ge et al., 1995), presumably through interaction with the negatively charged lipids in those complex systems. On the other hand, CD measurements on SP-D in the presence and in the absence of calcium were consistent with some changes in the secondary structure of the protein being produced by the calcium ions (Figure 7). It is worth noting that Ca^{2+}-induced conformational alterations in other proteins have led to changes in their solubilities (Maget-Dana & Ptak, 1995) or in their surface activities (Tsao et al., 1993). Accordingly, our surface tension measurements on aqueous solutions of SP-D indicated that the intrinsic tendency of SP-D to accumulate to the air−water interface was diminished by Ca^{2+} (Figure 1). Therefore, it is most likely that the effect of calcium to reduce the insertion of SP-D into the lipid monolayers was possibly due to Ca^{2+}-induced conformational changes in SP-D that attenuated the hydrophobic interactions between the protein and lipid monolayers.

Compressional Surface Pressure−Area Isotherms for SP-D/Lipid Monolayers. $\pi(F')$ curves for binary lipid monolayers of DPPC plus PI or PG that had been spread on subphases containing SP-A or SP-D were measured in order to test a potential role of lipid−protein interactions in removal of the unsaturated lipid from the mixtures. Such a mechanism of selective squeeze-out of non-DPPC components has been suggested to occur upon compression of the alveolar surface film in vivo during the breathing cycle (Watkins, 1968; Clements, 1977; Hildebran et al., 1979). The results from the measurements showed that, in the whole range of surface pressures studied, the curves for the DPPC−PI monolayers formed on the subphases containing SP-D or

SP-A (curves 2 and 3, Figure 8) were displaced toward higher areas compared to the curve for the lipid films spread on buffer alone (curve 1, Figure 8). This observation was consistent with some of the adsorbed SP-D or SP-A being incorporated into the DPPC−PI films that had been compressed to about 70 mN/m. The results in Figure 8 do not provide evidence whether or not some of the protein adsorbed initially to the lipid films was squeezed out during their compression. However, one may speculate that if SP-D or SP-A had been entirely excluded from the lipid monolayers during compression, then the isotherms for the films of DPPC−PI measured in the presence of protein (curves 2 and 3 in Figure 8) would have converged with that for the DPPC−PI films spread on protein-free subphases (curve 1, Figure 8) at higher surface pressures. The results in Figure 8 also suggested that if there were some exclusion of SP-D or SP-A during compression of the films, most likely it was not accompanied by selective removal of phospholipid molecules. Had any phospholipid been removed by SP-D, then the areas per phospholipid molecule for the DPPC−PI films formed on protein solutions, F', would have been lower than the areas, F^0 for DPPC−PI monolayers spread on the protein-free subphases.

CONCLUSIONS

Hydrophobic forces seemed to govern the interactions of SP-D in the subphase with monolayers of PC, PG, and PI spread at the air−water interface. Hydrophobicity of SP-D did not ensure binding of SP-D to PC and PG immobilized on thin-layer chromatography plates or microtiter wells (Ogasawara et al., 1992), but it favored the penetration of SP-D into phospholipid monolayers of PC and PG (Figures 3 and 5). No significant head group dependency was observed for interaction of SP-D with the monolayers of PC, PG, and PI (Figures 3 and 5), and this suggested an absence of specificity of the protein for the polar head groups of the lipids under the test conditions used here. In that, our results were in contrast to observations of preferential binding of SP-D to PI coated on thin-layer chromatography plates and microtiter wells (Ogasawara et al., 1992) or incorporated in DPPC liposomes (Persson et al., 1992). The discrepancy seen might be due to the different physical states of PI in these studies and a potential inadequacy of the measurements of surface pressure alone to evaluate more complex protein−lipid interactions than those due to predominantly hydrophobic forces.

Calcium ions diminished both the intrinsic surface activity of SP-D and its insertion into lipid monolayers (Figures 1 and 6). The ability of Ca^{2+} to affect the CD spectra of SP-D (Figure 7) suggested that the mechanism by which the cations altered the interactions of the protein with the phospholipid monolayers most likely involved conformational changes in SP-D. Surface pressure−area measurements failed to demonstrate a role of SP-D in selective removal of phospholipids from mixed DPPC−PI or DPPC−PG monolayers (Figures 8).

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