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Effects of a Surfactant-Associated Protein and Calcium Ions on the Structure and Surface Activity of Lung Surfactant Lipids†

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ABSTRACT: Previous studies have demonstrated that lung-specific proteins are associated with surfactant lipids, particularly the highly surface active subfraction known as tubular myelin. We have isolated a surfactant-associated protein complex with molecular weight components of 36 000, 32 000, and 28 000 and reassembled it with protein-free lung surfactant lipids prepared as small unilamellar liposomes. The effects of divalent cations on the structure and surface activity of this protein–lipid mixture were investigated by following (1) the state of lipid dispersion by changes in turbidity and by electron microscopy and (2) the ability of the surfactant lipids to form a surface film from an aqueous subphase at 37 °C. The protein complex markedly increased the rate of Ca^{2+}-induced surfactant–lipid aggregation. Electron microscopy demonstrated transformation of the small unilamellar liposomes (median diameter 440 Å) into large aggregates. The threshold Ca^{2+} concentration required for rapid lipid aggregation was reduced from 13 to 0.5 mM by the protein complex. This protein-facilitated lipid aggregation did not occur if Mg^{2+} was the only divalent cation present. Similarly, 5 mM Ca^{2+} but not 5 mM Mg^{2+} improved the ability of the protein–lipid mixture to form a surface film at 37 °C. Extensive aggregation of the surfactant lipids without protein by 20 mM Ca^{2+} or 20 mM Mg^{2+} did not promote rapid surface film formation. These results add to the growing evidence that specific Ca^{2+}–protein–lipid interactions are important in determining both the structure and function of extracellular lung surfactant fractions.

Pulmonary surfactant lipids are synthesized in the alveolar type II cell [reviewed in Van Golde (1976)]. Within the cell, these lipids are tightly packed into a membrane-bound organelle, the lamellar body (Gil & Reiss, 1973; Williams, 1982). After secretion of the lamellar body contents into the liquid layer lining the alveolar surface, at least some of the lamellae are transformed into tubular myelin, a characteristic latticelike structure found in fetal lung liquid (Williams, 1977), adult lungs (Williams, 1982), and lung washings (Gil & Reiss, 1973). This structure is thought to be the immediate precurosor of the monomolecular surface film which stabilizes the

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avleoli at low lung volumes [reviewed in Goerke (1974)]. In addition, other morphologically distinct forms of lung surfactant including multilamellar bilayer structures and small vesicles are seen in the alveolar space (Manabe, 1979) and in lung washing (Magoon et al., 1983). The factors which determine these different structural forms of extracellular surfactant are not well characterized.

In the complex life cycles of serum lipoproteins, specific apolipoproteins are important determinants of the structural organization and metabolism of the circulating lipoprotein complexes (Edelstein et al., 1979). Specific proteins in the surfactant system may have similar roles. Definitive classification of these proteins must wait further characterization, but glycoproteins with monomeric molecular weights of 30 000–40 000 have been identified in association with surfactant lipids in several different species including the dog (Sueishi & Benson, 1981), rat (Katyal & Singh, 1981), rabbit (Bhattacharyya & Lynn, 1980), cow (Sawada & Kashiwamata, 1977), and man (Shelley et al., 1982). There is immunochemical evidence that the major nonserum proteins associated with isolated surfactant are synthesized in the alveolar type II cell and are associated with the tubular myelin structure in the alveolar lining layer (Williams & Benson, 1981).

The structure of tubular myelin is reversibly dependent on calcium (Gil & Reiss, 1973; Benson et al., 1984). Whereas the functional advantage of this unique structure has not been established, only isolated surfactant equilibrated with calcium ions is able to rapidly form a surface film in vitro (Benson et al., 1984). These observations suggest that the structure and function of surfactant components are interrelated and are determined, at least in part, by specific protein–lipid–ion interactions.

The hypothesis that specific proteins promote the ability of lung surfactant lipids to rapidly form a surface film was suggested by the experiments of King & Clements (1972b). They found the lipid fraction extracted from surfactant did not form a surface film as rapidly as the isolated lipoprotein complex. More recently, reassembly experiments using isolated surfactant proteins and synthetic lipid mixtures have demonstrated improved lipid adsorption in the presence of surfactant proteins (King & Macbeth, 1977, 1981; King et al., 1983; Suzuki, 1982). In these studies, calcium was not required for lipid–protein reassembly but, as with isolated surfactant, calcium significantly improved the ability of the complex to form a surface film (King & Macbeth, 1981).

The present study was undertaken to investigate further the role of specific proteins and divalent cations in determining the structure–function relationships in lung surfactant.

Materials and Methods

Surfactant Preparations. Surfactant was isolated from lung washings by modification of a method previously reported in detail (King & Clements, 1972a). The lungs removed from exsanguinated dogs were degassed and lavaged 3 times at 4 °C with 1000 mL per lavage. The buffer used for the lavage and all subsequent experiments in this report was 5 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) and 100 mM NaCl, pH 7.4. The ionized calcium (Ca^{2+}) concentration of this buffer was less than 5×10^{-6} M (Radiometer F2112 Ca; Radiometer A/S, Copenhagen, Denmark). The pooled lung washings were spun at $150g_{av}$ for 15 min (Sorvall RC2-B) to remove cellular material. The supernatant was then spun at $20000g_{av}$ for 15 h (Beckman L3-40) using a type 15 rotor (Beckman Instruments), and the resulting pellet was dispersed in buffer containing 1.64 M sodium bromide. After equilibration for 1 h, this suspension was spun at $100000g_{av}$ for 4 h (Beckman L5-50B) in an SW-28 rotor (Beckman Instruments). The pellicle was resuspended in buffer and spun at $100000g_{av}$ for 1 h (Beckman L5-50B). This pellet was resuspended in double-distilled water and used as surfactant. All procedures, including the lavage, were performed at 4 °C, and the isolated material was stored at −15 °C.

Phospholipid concentration was calculated from the phosphorus content (Bartlett, 1959), and cholesterol was assayed by the method of Franey & Amador (1968). For protein determination (Lowry et al., 1951), 1% sodium dodecyl sulfate (SDS) was added to all samples, and bovine serum albumin was used as the standard.

Protein and Lipid Isolation. Surfactant in water, 10–15 mg of phospholipid/mL, was injected into a 50-fold excess by volume of 1-butanol (Sigrist et al., 1977) and was stirred at room temperature for 1 h. After centrifugation at $10000g_{av}$ for 20 min (Sorvall RC2-B), the supernatant was dried under vacuum at 40 °C, and the lipids were extracted (Folch et al., 1957). Phospholipid composition was determined by two-dimensional thin-layer chromatography (Poorthuis et al., 1976).

The precipitate from the 1-butanol extraction was dried under nitrogen and washed twice in 20 mL of buffer containing 20 mM octyl β-D-glucopyranoside. After centrifugation at $100000g_{av}$ for 1 h (Beckman L5-50B), the pellet was dispersed in 0.3 M lithium diiodosalicylate and 0.05 M pyridine (pH 8.4) on ice, diluted with an equal volume of water, and mixed with a volume of 1-butanol equal to the aqueous phase (King & Macbeth, 1979). A total of nine 1-butanol–water partitions were performed to lower the detergent concentration in the aqueous phase. The final, lower, aqueous phase was lyopilized for 15 h, taken up in 2 mL of buffer, and spun at $100000g_{av}$ (Beckman L5-50B) to remove any remaining insoluble material. The lithium diiodosalicylate concentration in the final sample, calculated from a molar extinction coefficient of 4×10^3 at 323 nm (Marchesi & Andrews, 1971), was less than 10 μM.

Polyacrylamide slab gel electrophoresis (1.5 mm × 105 mm × 150 mm) was performed by using buffers containing sodium dodecyl sulfate (SDS) (Laemmli, 1970). 2-Mercaptoethanol, 5% by volume, was added to each of the samples. The reactivity of samples from each step in the purification procedure against anti-dog serum IgG (Cappel Laboratories, Philadelphia, PA) and anti-dog surfactant IgG prepared in our laboratory (Sueishi & Benson, 1981) was assessed by rocket immunoelectrophoresis and immunoblot electrophoresis.

Liposome Preparation. Liposomes were prepared in a French pressure cell (3-mL capacity: Aminco, Silver Spring, MD) by using methods previously described in detail (Hamilton et al., 1980). An aliquot of the extracted surfactant lipids in chloroform–methanol (2:1 v/v) was dried under nitrogen and hydrated in buffer (2–3 mg of phospholipid/mL) for 1 h at room temperature. This mixture was expressed from the pressure cell 3 times at a pressure of 20 000 psi at room temperature. The eluant was spun at $100000g_{av}$ for 30 min (Beckman L5-50B), and the pellet, containing 10–15% of the starting phospholipid in large multilamellar forms, was discarded. Liposomes in the supernatant were stored at 4 °C and used within 96 h of preparation.

Turbidity Measurements. Turbidity, as a measure of liposome aggregation and/or fusion, was measured at 400 nm in a Beckman DU Model 2400 spectrophotometer (Beckman Instruments Inc., Fullerton, CA). All experiments were

performed at 37 °C with a phospholipid concentration of 200 μg/mL. $CaCl_2$, $MgCl_2$ (both analytical grade from Mallinckrodt Inc., Paris, KY), or ethylenediaminetetraacetic acid (EDTA) (Eastman Kodak Co., Rochester, NY) was added to the samples in a 1-mL glass cuvette from 100 mM stock solutions, the samples were mixed, and the change in turbidity was recorded.

In a second series of experiments, sequential aliquots of $CaCl_2$ or $MgCl_2$ were added to the samples every 4 min, and the turbidity was measured just before the next increase in ion concentration. The divalent ion concentration corresponding to the maximal rate of change in turbidity was taken as the threshold concentration for cation-induced aggregation (Ohki et al., 1982). Human albumin (Cutter Laboratories, Berkeley, CA) dialyzed against the standard buffer was used in control experiments.

Electron Microscopy. Extracted lipid liposomes, 1–2 mg of phospholipid/mL in the standard buffer, were warmed to 37 °C, and either EDTA or $CaCl_2$ was added from 100 mM stock solutions. In all lipid–protein experiments, the protein was added to the liposomes at 37 °C and incubated for 10 min prior to the addition of divalent cations or EDTA. Negative staining of the different liposome mixtures was done after 1 h of incubation at 37 °C, and electron micrographs of representative images were taken the same day with a Siemens 101 electron microscope (Siemens Medical Industrial Groups, Iselin, NY) (Hamilton et al., 1980). The size distribution of freshly prepared liposomes was determined by measuring 200 particles on photomicrographs magnified 180000×.

Surface Activity. The ability of the extracted lipid and lipid–protein samples to adsorb and spread at an air–water interface from a stirred subphase was tested at 37 °C. Samples containing 340 μg of phospholipid in 2 mL were incubated at 37 °C for 1 h in buffer containing Ca^{2+}, Mg^{2+}, or EDTA. Thirty-four milliliters of buffer (containing added EDTA, $CaCl_2$, or $MgCl_2$) was stirred at 120 rpm in a circular Teflon trough 6.2 cm in diameter. Stirring in the trough was stopped, the surface was cleaned by gentle aspiration of 2 mL from the surface, and the sample was carefully layered on the bottom of the trough with a Pasteur pipet. Stirring was restarted, and the surface tension was continuously measured by a platinum dipping plate suspended from a strain gauge (Statham Gold Cell, Hato Ray, Puerto Rico). The signal was amplified and displayed on a chart recorder. Surface pressure was calculated by subtracting the measured surface tension from the surface tension of the buffer alone at 37 °C (70 mN/m). The subphase phospholipid concentration was 10 μg/mL, unless otherwise stated.

Results

Preparation of Surfactant Lipids and Proteins. The surfactant isolated by the methods described was a lipoprotein complex containing 12% protein by weight. The yields given in this section are the average from two different preparations. Following the butanol extraction, 6% of the total surfactant-associated proteins were recovered with the surfactant lipids, and 88% were recovered as a fine precipitate. The majority of the proteins, accounting for 67% of the total surfactant-associated proteins and including all the immunoreactive serum proteins, were separated from the more hydrophobic, nonserum, surfactant-associated proteins by their solubility in 20 mM octyl β-D-glucopyranoside. The more hydrophobic proteins were solubilized in lithium diiodosalicylate which was then partitioned into an alcohol phase. In the final protein preparation, a major band with an apparent monomeric molecular weight of 32 000 and two minor bands with apparent molecular weights of 36 000 and 28 000 were resolved by gradient slab gel electrophoresis and Coomassie Blue staining (Figure 1). This preparation had no reactivity with antibodies to whole dog serum on rocket immunoelectrophoresis or immunoblot electrophoresis.

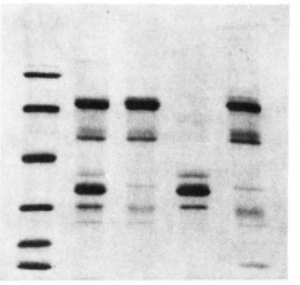

FIGURE 1: SDS–polyacrylamide gel electrophoresis. The polyacrylamide concentration gradient ranged from 7.5% to 16%. (A) Low molecular weight standards (Pharmacia Fine Chemicals, Piscataway, NJ) (from top to bottom): phosphorylase *b* (94K), albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), trypsin inhibitor (20.1K), and α-lactalbumin (14.4K); (B) isolated surfactant, 15 μg of protein; (C) octyl β-D-glucopyranoside soluble fraction (predominantly serum proteins), 10 μg of protein; (D) purified protein preparation (nonserum surfactant proteins), 5 μg of protein; (E) dog serum (Cappel Laboratories, Philadelphia, PA), 14 μg. The gel was stained with Coomassie Brilliant Blue.

Table I: Phospholipid Composition[a]

	composition
origin	0.3 ± 0.2
unknown	0.7 ± 0.5
lysophosphatidylcholine	0.4 ± 0.4
sphingomyelin	1.0 ± 0.2
phosphatidylserine[b] phosphatidylinositol[b]	3.4 ± 1.0
phosphatidylglycerol	14.2 ± 0.9
phosphatidylcholine	76.6 ± 1.7
phosphatidylethanolamine	2.2 ± 0.8
bismonoacylglycerol phosphate	1.1 ± 0.5
front	0.1 ± 0.1

[a] Values (% phosphorus) are mean ± SE for three different animals. [b] Combined composition of phosphatidylserine plus phosphatidylinositol.

The surfactant lipids, extracted first into butanol and then into chloroform and methanol, contained less than 0.05% protein by weight. No protein in the 30 000–40 000-dalton range was detectable in this lipid preparation on Coomassie Blue stained polyacrylamide gels. The phospholipid composition of the extracted lipids is shown in Table I. Anionic phospholipids accounted for 18% of the total. The phospholipid compositions of the extracted lipids and of the liposomes prepared from them were not significantly different. In addition to phospholipid, the liposomes contained 8% cholesterol by weight. The median diameter of the liposomes prepared in the standard buffer was 440 Å (range 220–1440 Å). The electron microscopic appearance of the liposomes did not change during storage at 4 °C for 4 days.

Turbidity Measurements. Tracings from representative experiments showing the change in turbidity after the addition of Ca^{2+} or Mg^{2+} to the lipids or lipoprotein mixtures are shown in Figure 2. Each experiment was repeated 3–7 times. The absolute change in turbidity varied with different lipid and protein preparations, but the relative effects of the protein, Ca^{2+}, and Mg^{2+} on liposome aggregation were very reproducible. Without surfactant protein, 5 mM Ca^{2+} induced slow aggregation of the liposomes. The rate and extent of aggregation of the extracted liposomes were increased at higher Ca^{2+}

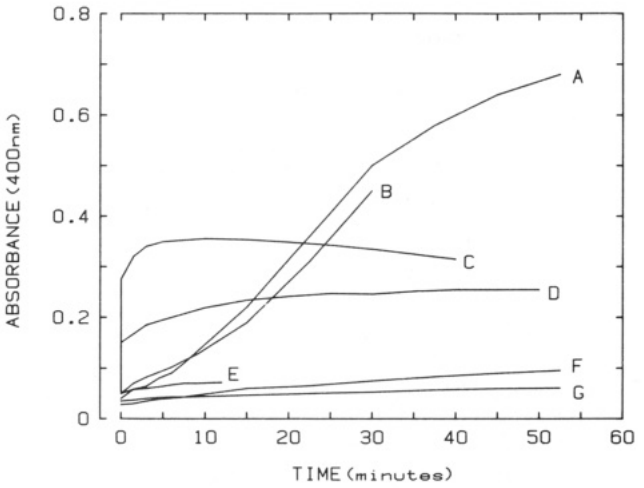

FIGURE 2: Change in absorbance (400 nm) at 37 °C. Liposomes (200 μg/mL) were incubated alone or with added surfactant protein for 10 min at 37 °C in a 1-mL glass cuvette. Divalent cations were then added (0 min) from 100 mM stock solutions. The samples were mixed, and absorbance was continuously monitored. (A) Lipids alone, 20 mM Ca^{2+}; (B) lipids alone, 20 mM Mg^{2+}; (C) 5 to 1 phospholipid to protein (w/w), 5 mM Ca^{2+}; (D) 20 to 1 phospholipid to protein (w/w), 5 mM Ca^{2+}; (E) 20 to 1 phospholipid to protein (w/w), 5 mM Mg^{2+}; (F) lipids alone, 5 mM Ca^{2+}; (G) lipids alone, 5 mM Mg^{2+}.

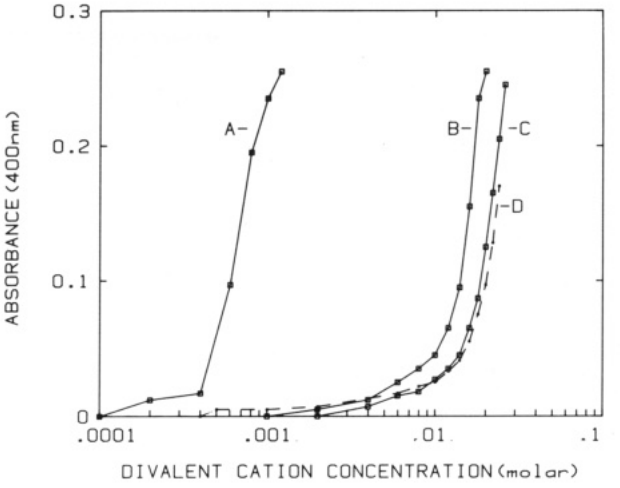

FIGURE 3: Turbidity (absorbance 400 nm) as a function of Ca^{2+} or Mg^{2+} concentration. The concentration of divalent cation was changed every 4 min. The concentration corresponding to the maximal increase in turbidity was taken as the threshold concentration for aggregation. The phospholipid concentration in all samples was 200 μg/mL, and the phospholipid to protein ratio in (A) and (D) was 20 to 1 (w/w). (A) lipid–protein, Ca^{2+}; (B) lipid only, Ca^{2+}; (C) lipid only, Mg^{2+}; (D) lipid–protein, Mg^{2+}.

concentrations, but, in the absence of protein, maximum absorbance values were not reached in less than 60 min (Figure 2). Mg^{2+} caused a similar slow aggregation of the extracted lipid liposomes.

The initial rate of aggregation in 5 mM Ca^{2+}, but not Mg^{2+}, was markedly enhanced by the isolated protein preparation. With phospholipid to protein ratios of 20 to 1 (w/w), extensive aggregation occurred within seconds in contrast to the very slow aggregation of the extracted lipids without protein. Decreasing the phospholipid to protein ratio (w/w) from 20 to 1 to 5 to 1 caused further aggregation in 5 mM Ca^{2+}. In control experiments, the surfactant protein did not cause any liposome aggregation in the presence of EDTA, and the rate of Ca^{2+}-induced aggregation was not affected by albumin.

The relationship between the divalent cation concentration and the change in tubidity is shown in Figure 3. The ion concentration corresponding to the maximum rate of absorbance increase of the extracted lipid liposomes was 13 and 15 mM for Ca^{2+} and Mg^{2+}, respectively. The protein reduced the Ca^{2+} threshold to 0.5 mM but did not enhance aggregation if Mg^{2+} was the only divalent cation present.

Electron Microscopy. Electron micrographs of representative negatively stained samples of the liposome mixtures are shown in Figure 4. Calcium (3 mM) caused some aggregation of the extracted lipid liposomes without added protein after a 1-h incubation at 37 °C (Figure 4, middle left panel). Extensive aggregation of the extracted lipids occurred with calcium concentration of 10 mM (Figure 4, bottom left panel) or greater after 1 h at 37 °C. The electron micrographs did not show any change in the liposome structure when the lipids were incubated with the surfactant–protein complex in the absence of divalent cations (Figure 4, top right panel), but the liposome aggregation induced by Ca^{2+} was markedly enhanced by the protein complex (Figure 4, bottom right panel). It was not possible to distinguish between liposome aggregation and fusion using this technique.

Surface Activity. Representative tracings showing the ability of the lipid or lipoprotein mixtures to form a surface film are shown in Figures 5 and 6. As shown in Figure 5D, the surfactant lipids form a surface film very slowly in the

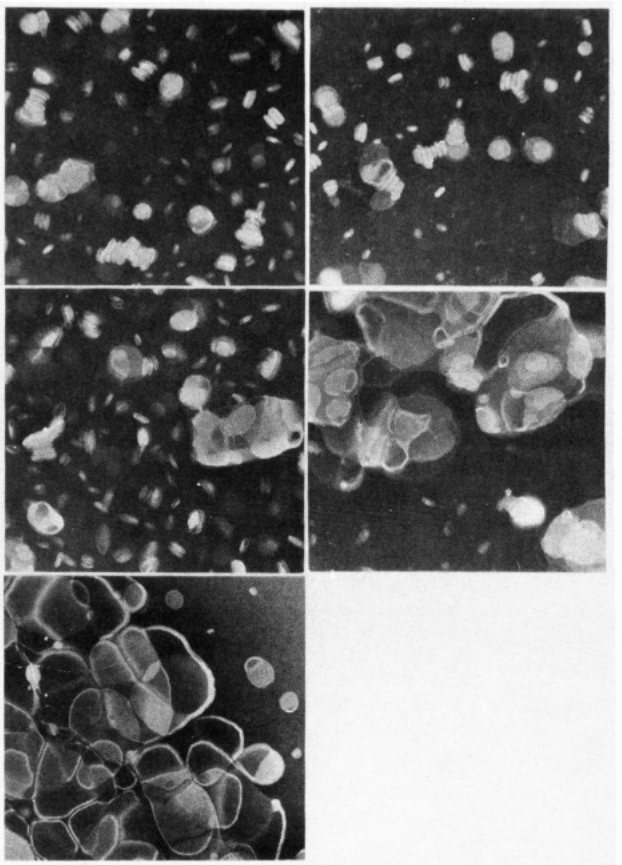

FIGURE 4: Electron micrographs (54000×) of liposomes prepared with a French pressure cell and incubated for 1 h at 37 °C. (Upper left) Extracted lipid liposomes, 1 mM EDTA; (middle left) extracted lipid liposomes, 3 mM Ca^{2+}; (bottom left) extracted lipid liposomes, 10 mM Ca^{2+}; (upper right) liposomes plus surfactant proteins (10 to 1 w/w), 1 mM EDTA; (lower right) liposomes plus surfactant proteins (10 to 1 w/w), 3 mM Ca^{2+}.

absence of divalent cations. Adsorption of the extracted lipids was promoted by incubating the samples in 5 mM Ca^{2+} (Figure 5C) or 5 mM Mg^{2+} (Figure 5B) for 1 h at 37 °C prior to testing their surface activity. Despite the extensive liposome aggregation induced by 20 mM Ca^{2+} by 60 min (Figure 2 and

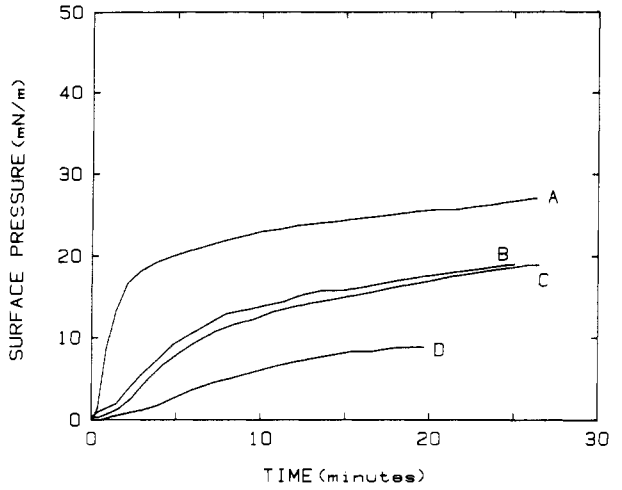

FIGURE 5: Change in surface pressure as a function of time for representative samples of the extracted lipids without added protein. The samples were incubated for 1 h in buffer containing 1 mM EDTA, 5 mM $CaCl_2$, or 5 mM $MgCl_2$ and then deposited beneath a clean surface of the same buffer (see text for details). The equilibrium surface pressure at 37 °C is 45 mN/m. (A) 120 μg of phospholipid/mL, 5 mM Ca^{2+}; (B) 10 μg/mL, 5 mM Mg^{2+}; (C) 10 μg/mL, 5 mM Ca^{2+}; (D) 10 μg/mL, 1 mM EDTA.

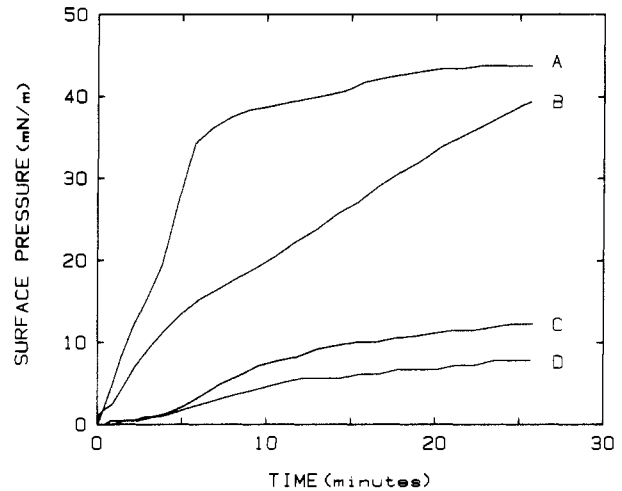

FIGURE 6: Change in surface pressure as a function of time for representative samples containing 10 μg of phospholipid/mL plus (A) surfactant protein (5 to 1, phospholipid to protein, (w/w) and 5 mM Ca^{2+}, (B) surfactant protein (20 to 1, phospholipid to protein, w/w) and 5 mM Ca^{2+}, (C) surfactant protein (20 to 1, phospholipid to protein, w/w) and 5 mM Mg^{2+}, or (D) surfactant protein (20 to 1, phospholipid to protein, w/w) and 1 mM EDTA.

unpublished electron micrographs), the rate of surface film formation was not significantly increased over that observed with 5 mM Ca^{2+} (data not shown). Increasing the phospholipid concentration in the subphase from 10 to 120 μg/mL improved the initial adsorption rate (Figure 5A), but the surface pressure of any of the extracted lipid samples tested in buffers containing 0–40 mM Ca^{2+} or Mg^{2+} did not exceed 25 mN/m at 30 min. The equilibrium surface pressure expected for both the extracted lipid and lipid–protein complexes is 44–46 mN/m at 37 °C. This represents the maximum surface pressure of an uncompressed surface film of the surfactant lipids.

The surfactant-associated proteins markedly enhanced the rate of surface film formation when the lipid–protein sample was incubated in 5 mM Ca^{2+} (Figure 6A,B) but not in 5 mM Mg^{2+} (Figure 6D). Although considerable variability between different preparations was seen, the surface pressures at 5, 15, and 30 min were significantly greater with the lipoprotein

Table II: Surface Pressure[a]

	time (min)		
	5	15	30
EDTA (5)	2 ± 1	6 ± 1	9 ± 1
EDTA + protein (20:1) (2)	3 ± 1	7 ± 1	9 ± 1
5 mM Ca^{2+} (7)	5 ± 1	13 ± 1	18 ± 1
5 mM Ca^{2+} + protein (20:1)[b] (4)	8 ± 2	17 ± 3	36 ± 3[c]
5 mM Ca^{2+} + protein (5:1)[b] (3)	18 ± 7[c]	34 ± 5[c]	44 ± 1[c]

[a] Values (in millinewtons per meter) are means ± SE. Samples were incubated for 1 h at 37 °C in 1 mM EDTA or 5 mM Ca^{2+} and then placed below a clean surface and the change in surface pressure recorded. The subphase phospholipid concentration was 10 μg/mL (see text for details). Values in parentheses are the number of experiments performed. [b] Ratio of phospholipid to protein by weight. [c] $P < 0.05$ compared to lipids alone in 5 mM Ca^{2+} at an equivalent time point (unpaired t test).

mixture than with the lipids alone in the presence of 5 mM Ca^{2+} (Table II). The rate of surface film formation was dependent on the mass of protein added to the liposomes (Figure 6A,B), but the stoichiometry of the reassembled complex was not determined in these experiments. The delipidated protein, prepared as described, had minimal intrinsic surface activity. With a subphase concentration of 2 μg/mL, an equilibrium surface pressure of 14 mN/m was reached in 40 min. We note however that the delipidated protein prepared by methods very similar to those used in this study forms large molecular weight complexes in aqueous buffers (King et al., 1983; unpublished observations). These protein complexes are not fully dissociated by SDS in the absence of reducing agents, suggesting strong protein–protein interactions (Katyal & Singh, 1981). Such interactions may influence both lipid–protein reassembly (Ritter & Scanu, 1977) and the assessment of protein surface properties. In the control experiments, albumin, with or without Ca^{2+}, did not enhance the ability of the surfactant lipids to form a surface film.

Discussion

Rapid surface film formation from an aqueous subphase is thought to be critical for normal lung surfactant function (King & Clements, 1972b). Tubular myelin, a structurally unique subfraction of extracellular lung surfactant, may serve this function (Goerke, 1974). The conversion of lamellar bodies to tubular myelin in vitro is dependent on Ca^{2+} (Sanders et al., 1980) and may involve the highly ordered aggregation or fusion of lipid bilayers (Sanderson & Vatter, 1977).

We have shown that a surfactant protein preparation markedly enhances the calcium-induced aggregation of surfactant lipid bilayers. This protein preparation is similar in electrophoretic mobility and immunological reactivity to the major nonserum protein associated with canine surfactant prepared by ion-exchange chromatography (Sueishi & Benson, 1981). The improved resolution of gradient electrophoresis has shown at least three components of different molecular weight. The same electrophoretic appearance has been described for the major nonserum proteins of rat surfactant (Katyal & Singh, 1981). It remains to be established whether this heterogeneity represents differences in amino acid and carbohydrate composition and whether all the components of this complex are required for the full expression of function. In a limited number of experiments using differential centrifugation, we have found that greater than 85% of the protein is associated with the aggregated lipid in the presence of 3 mM Ca^{2+}. This finding is consistent with the results reported for reassembly of this protein with model phospholipid mixtures (King & Macbeth, 1981; King et al., 1983). Ca^{2+} was not required for the interaction of the protein and mixtures of

phosphatidylcholine and phosphatidylglycerol, but Ca^{2+} did affect the stoichiometry and structure of the reassembled complex. Rapid aggregation of the liposomes occurred only when both surfactant protein and 3 mM Ca^{2+} were present in the reaction mixture (King et al., 1983).

Lung surfactant is a complex mixture of lipid species. With the exception of dipalmitoylphosphatidylcholine, the function of the minor lipid components is poorly understood. To further investigate the interaction of surfactant proteins and divalent cations with the lipid components of lung surfactant, we have used the total lipid fraction extracted from isolated surfactant for liposome preparation. The lipid composition was similar to that previously reported for dog surfactant (King & Clements, 1972b) except for an increased proportion of phosphatidylglycerol in our preparation. The reason for this difference is unknown but may reflect the small number of determinations in the present study.

The electron microscopic images show the unilamellar liposomes were transformed into large aggregates by Ca^{2+}. The threshold concentration of Ca^{2+} required for aggregation was reduced from 13 to 0.5 mM by the protein preparation. The potentially different processes of bilayer aggregation and fusion could not be distinguished by the methods used here. The ionic composition of the alveolar space has not been extensively studied, but a Ca^{2+} concentration of 1.6 mM, determined by an ion-selective electrode, has been reported for adult rabbit lungs (Nielson, 1984). Our results show that rapid and extensive aggregation of surfactant lipid bilayers occurs at this Ca^{2+} concentration only when specific proteins are present. These findings support the hypothesis that, in addition to calcium ions (Gil & Reiss, 1973; Sanders et al., 1980; Benson et al., 1984), specific proteins also modify the state of dispersion of surfactant lipids (Williams, 1977; King, 1983).

The extent of ion binding to phospholipid head groups is one of the important determinants of bilayer aggregation (Ekerdt & Papahadjopoulos, 1982). Ca^{2+} has been shown to neutralize the surface charge, decrease the surface hydration, increase the phase transition temperature, and cause lipid segregation of phospholipid bilayers. The net result of these effects is to promote the close apposition of bilayers necessary for aggregation and fusion [reviewed in Rand (1981)]. Phosphatidylcholine (PC), the major phospholipid of lung surfactant, binds Ca^{2+} weakly at physiological pH and aggregates only at concentrations of Ca^{2+} approaching 100 mM (Hauser et al., 1975). Anionic lipids, predominantly phosphatidylglycerol (PG), made up 18% by weight of the total lipid in our liposomes. The reported intrinsic association constant of Ca^{2+} to PG is 8.5 M^{-1} (Lau et al., 1981), and rapid aggregation and fusion of PG liposomes occur in 10 mM Ca^{2+} (Rosenberg et al., 1983). The slow aggregation of the surfactant lipid liposomes with high Ca^{2+} concentrations is therefore not unexpected, but the mechanism by which the surfactant protein preparation promotes the aggregation of surfactant lipids is not known at present. The sensitivity and specificity of this effect for Ca^{2+} suggest that specific Ca^{2+}–protein interactions may be involved.

The ability of the protein to enhance the adsorption of surfactant lipids to the air–water interface was also specifically dependent on Ca^{2+}. The low solubility of phospholipids in water (Smith & Tanford, 1972) and the relatively low calculated rate of monomeric transfer of phosphatidylcholine between liposomes (McLean & Phillips, 1981) suggest that particulate transfer of phospholipids from the subphase to the surface film would be necessary to account for the adsorption rate of surfactant measured in vitro and calculated in vivo. The state of lipid dispersion has been shown to influence the rate of surface film formation for both synthetic lipid mixtures (Goerke, 1981) and extracted lung lipids (Notter, 1983). We found that the surfactant-associated protein enhanced lipid aggregation and promoted the spontaneous formation of a surface film in processes that were specifically dependent on Ca^{2+}. In contrast, aggregation of the protein-poor lipids by high Ca^{2+} concentrations was not associated with similarly improved adsorption characteristics. This suggests that phospholipid aggregation alone is not sufficient for rapid film formation. We note, however, that because turbidity reflects both particle size and refractive properties, a strict relationship between the measured turbidity and extent of lipid aggregation cannot be assumed. Whether the increase in adsorption rate observed with the protein–lipid complex is dependent on specific structural changes within the lipid aggregates is not known at present.

Whole lung surfactant will also rapidly form a surface film when Ca^{2+} but not Mg^{2+} is present in the subphase (Notter et al., 1983). Although our results suggest this specificity may be due to surfactant-associated proteins, extracted surfactant lipids dispersed in 0.15 M NaCl (Notter et al., 1983; Kobayashi & Robertson, 1983) have been shown to adsorb rapidly without added Ca^{2+} when tested at sufficiently high subphase lipid concentrations. In these studies, the extracted lipids contained 1% protein, but the characteristics of this residual protein were not reported. As only a small fraction of the total sample is required to form a surface film at equilibrium pressure and the adsorbing particles may be heterogeneous in structure and composition, the surface activity results presented here and elsewhere should be interpreted cautiously.

King & Clements (1972c) have postulated that the adsorption process is rate limited by the energy barrier associated with hydrocarbon–water interactions occurring during the release of phospholipid from aggregates into the surface film. Ca^{2+} binding, either to the protein directly or to the anionic phospholipids in a protein-facilitated fashion, would reduce the surface hydration of the lipid bilayers (Hauser et al., 1975; Ekerdt & Papahadjopoulos, 1982) and potentially decrease bilayer stability. Both mechanisms could enhance the entry of lipid molecules into the surface film. The finding that surfactant dehydrated under nitrogen spreads more rapidly on water than fully hydrated samples of the same material (Morley et al., 1978) supports the proposed importance of surface hydration as a barrier to adsorption.

We have shown that surfactant-associated proteins in the presence of Ca^{2+} exert major changes on the physical properties of surfactant lipids in vitro. These results imply a significant physiological role for these proteins, but further studies will be required to understand the events occurring at the protein–lipid–solvent interface which account for the unusual structural and surface properties of tubular myelin.

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