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Calcium ions and interactions of pulmonary surfactant proteins SP-B and SP-C with phospholipids in spread monolayers at the air/water interface

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Abstract

Spread monolayers containing hydrophobic pulmonary surfactant protein, SP-B or SP-C, or SP-B/SP-C (2:1, w/w), alone or mixed with dipalmitoylphosphatidylcholine (DPPC) or dipalmitoylphosphatidylglycerol (DPPG), were formed on saline subphases containing calcium ions. Surface pressure-area characteristics of the films of the proteins were not affected by the presence of Ca^{2+} in the subphase. Calcium ions did not alter the surface properties of the binary and ternary films of DPPC plus either SP-B, or SP-C, or SP-B/SP-C (2:1, w/w). Surface pressure-area isotherms for the spread films of DPPG plus hydrophobic surfactant protein were Ca^{2+}-dependent. The exclusion pressures of SP-B, SP-C and SP-B/SP-C (2:1, w/w) from protein-DPPG films in the presence of calcium were lower than the exclusion pressures in the absence of Ca^{2+}. The divalent cation appeared to suppress the ability of SP-C and SP-B/SP-C (2:1, w/w) to remove phospholipid during squeeze-out from their mixed films with DPPG. The effects of Ca^{2+} on the monolayers of DPPG plus hydrophobic surfactant proteins were consistent with calcium producing diminished lipid–protein interactions, possibly resulting from Ca^{2+}-induced changes in the ionization state and molecular packing of DPPG.

Keywords: Surface balance; Langmuir film; Dipalmitoylphosphatidylcholine; Dipalmitoylphosphatidylglycerol; Lipid–protein interaction

1. Introduction

Lung surfactant, a mixture of lipids and proteins, is found in the aqueous layer which covers the respiratory epithelium. Lung surfactant contributes to pulmonary stability by lowering the surface tension in the alveoli [1]. Calcium, which is an endogenous component of lung surfactant, has an effect on surfactant structure and function. Ca^{2+} is required for in vitro assembly of tubular myelin, a morphological form of extracellular surfactant which probably has relevance to the formation of the putative surface film in alveoli in vivo [2]. Optimal adsorption of natural lung surfactant [3–6] and lipid extracts of surfactant [7] required the presence of calcium ions in the subphase. The ability of the specific surfactant proteins, SP-A, SP-B and SP-C, to enhance the rate of adsorption of phospholipids from vesicles in the subphase into the air/water interface was Ca^{2+}-dependent [8–12]. Different mechanisms have been proposed for the effect of Ca^{2+} on the surface activity of pulmonary surfactant [6,11,13]. Ca^{2+} may affect lipid–protein interactions in the surfactant lipoprotein complexes through conformational changes in the proteins and through changes in the charge and hydration state of the polar head groups of the phospholipids [11,13]. Ca^{2+} may also act in a more general way to neutralize the surface charges of lipoprotein vesicles and hence to modify the electrostatic forces between the vesicles in the subphase and the surface film [6].

In the present study, we used model monolayer systems to examine how calcium ions in the subphase affect the properties of spread films of the hydrophobic pulmonary surfactant proteins, SP-B, SP-C, and SP-B/SP-C (2:1, w/w). The effect of Ca^{2+} on the lipid–protein interactions in mixed monolayers of the hydrophobic pulmonary surfactant proteins with dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylglycerol, which represent the principal lung surfactant phospholipids, was also investigated.

SP-B is a disulfide-linked dimer of 79-amino acid residue monomers which contains regions of amphipathic α-helix and has a net positive charge. Porcine SP-B has 9

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positively charged side chains at physiological pH and two negatively charged ones [14]. SP-C is an extremely hydrophobic 35-amino acid residue protein which has two palmitoyl chains covalently bound through thiol esters to cysteines at positions 5 and 6. It has a 23-residue C-terminal α-helical portion and also has a net positive charge. Porcine SP-C has three positively charged side chains at physiological pH and no negatively charged ones [15]. Both proteins may have a role in the dynamics of surfactant in the aqueous lining layer of the alveolar spaces.

A preliminary report of the work described in this paper has been published [16].

2. Materials and methods

Dipalmitoylphosphatidylcholine (DPPC) was purchased from Sigma, St. Louis, MO, and dipalmitoylphosphatidylglycerol (sodium salt) (DPPG) from Avanti Polar Lipids, Alabaster, AL. The lipids were pure as determined by thin-layer chromatography and were used as received. Sodium chloride and calcium chloride, ACS-reagent grade, were obtained from Fisher Scientific, Ottawa, ON.

Surfactant proteins SP-B and SP-C were prepared from porcine lung lavage fluid as described previously [17]. Gel exclusion chromatography on Sephadex LH-60 (chloroform/methanol, 1:1 (v/v) containing 1% (by volume) of 0.1 M HCl, as opposed to 5% of 0.1 M HCl in the previous paper [17]) was used for isolation and purification of the hydrophobic proteins from the lipid extract. On SDS-polyacrylamide gel electrophoresis (16% gels) under non-reducing conditions, SP-B yielded a major band at about 18 kDa and a minor one at about 29 kDa. SP-C showed one band at about 5 kDa. There was little lipid contamination of the proteins, less than 0.5 mol phospholipid per mol SP-B (dimer) and less than 0.05 mol phospholipid per mol SP-C (monomer), as indicated by the analysis of phosphorus in the protein preparations [18,19].

Surface pressure-area measurements were performed on a Langmuir trough, Applied Imaging (Dukesway Team Valley, Gateshead, Tyne and Wear, UK), which employed a continuous teflon ribbon barrier. Surface tension was measured by the Wilhelmy plate method with a roughened platinum plate. Known amounts of protein and lipid were spread from a common solvent (chloroform/methanol, 3:1 (v/v)) into the air/water interface to form mixed monolayers. The estimation of protein in the spreading solutions was done by the fluorescamine assay [20] and phospholipid phosphorus was determined by a modification of the method of Bartlett [18,19]. The liquid subphase contained 150 mM NaCl plus 2 mM $CaCl_2$ in deionized doubly-distilled water. Immediately before each experiment the pH of the subphase was adjusted to 7 with 0.01 M NaOH, and it did not change by more than 1 pH unit during surface pressure-area measurements. This concentration of $CaCl_2$ was in the range of the Ca^{2+} level reported for the extracellular aqueous lining layer of lung alveoli [21]. The ionic composition of the alveolar space has not been extensively studied, but the alveolar subphase pH in the lungs of anesthetized rabbits has been measured, pH 6.92 [22]. The surface pressure behavior for DPPC is unaffected by the pH of subphase over a large range of pH values [23]. The ionization state of DPPG monolayers appears to be independent of pH for pH > 4 when the subphase ionic strength corresponded to > 10 mM NaCl [36]. Measurements in this laboratory showed that the isotherms for either SP-B or SP-C were identical at pH 5.8 (unbuffered) and pH 7 (25 mM Hepes) for subphase composition of 150 mM NaCl. Therefore, no buffer was used in order to provide a simple ion composition of the subphase for the measurements of the effect of calcium on binary and ternary interactions in the spread monolayers. The compression isotherms were obtained by continuous reduction of the film area at a rate of 40 cm^2/min. The total compression between a maximal area of 500 cm^2 to a minimal area of 100 cm^2 took 10 min. The measurements were conducted at $22 \pm 2°$ C. Whereas the surfactant in mammals functions at 37° C, that found in air-breathing poikilotherms operates over a range of temperatures including 22° C. In this temperature interval both DPPC and DPPG are below their gel to liquid crystalline transition temperature ($T_c = 41°$ C). While there may be some quantitative differences between measures of their interactions with the hydrophobic proteins or calcium ions at 22° C and 37° C, the major qualitative factors influencing the properties of the lipid-protein monolayers are likely to be the same at the two temperatures.

The initial compositions of the lipid-protein monolayers were given by the 'residue' fraction of the amino acid residues of the protein, $X_r = N_r/(N_r + N_l)$, where N_r is the number of protein amino acid residues, and N_l is the number of lipid molecules spread in each monolayer. For all calculations a molecular weight of 8700 was used for monomeric SP-B (79 amino acid residues) and a molecular weight of 4186 for SP-C (35 amino acid residues plus two palmitate chains) [15]. The mean areas in the lipid-protein films, A_{mean}, were expressed as mean area per 'residue', where 'residue' denoted a phospholipid molecule or a protein amino acid residue. In the ternary films of SP-B/SP-C plus phospholipid the ratio between the two proteins was 2:1 (w/w). This ratio, consistent with findings that SP-B is the predominant (by mass) protein of the two hydrophobic surfactant proteins [24,25], corresponded to 69.7% amino acid residues of SP-B and 30.3% amino acid residues of SP-C in the protein mixture. Similar measurements were performed with protein-lipid monolayers containing SP-B/SP-C of 1:1 ratio (w/w). The results, not included in this paper, showed that Ca^{2+} had qualitatively similar effects on the monolayers of SP-B/SP-C, 1:1, plus phospholipid as those shown here for the 2:1 ratio.

3. Results and discussion

3.1. Monolayers of surfactant proteins SP-B, SP-C and SP-B / SP-C

The surface pressure (π)-area per amino acid residue isotherms for the single-component monolayers of SP-B (curve 1), SP-C (curve 2), and for their mixture SP-B/SP-C (2:1, w/w) (curve 3) in the presence of calcium are plotted in Fig. 1 (continuous lines). Results from similar measurements on the protein monolayers in the absence of calcium are shown by closed circles in Fig. 1. The isotherm for each protein monolayer in the presence of Ca^{2+}, continuous line in Fig. 1, was an average result (± 0.01 nm^2/amino acid residue) from two separate measurements with one protein preparation. Calcium in the subphase did not influence those monolayer characteristics of the protein monolayers, such as area per amino acid residue and surface pressure at maximum compression. In accordance with these observations, earlier studies have shown that the circular dichroism spectra of transferred monolayers of SP-B and SP-C were independent of the presence of calcium in the subphase [26]. Ca^{2+} binding to other protein monolayers altered their intrinsic interfacial properties [27]. In that case, Ca^{2+} in the subphase presumably caused charge neutralization and conformational changes of the protein [27]. The isotherm for the monolayers of SP-B or SP-C, or their combination exhibited a kink point at a surface pressure of about 20–25 mN/m. The mechanistic origin of this inflection in the isotherms is not known. An inflection or, in some cases, a flat plateau has been observed at similar pressures in spread films of SP-C [28,29] and synthetic α-helical polypeptides [30]. This transition in the films of polypeptides has been interpreted in terms of a transformation from a monolayer to a bilayer [30], or in terms of changes in the degree of packing density of the α-helices in the monolayer plane [31]. Ellipsometric measurements on monolayers of SP-C have shown a continuous increase in the thickness of the films and this has been accounted for by a reversible formation of bilayer/multilayer structures at the interface [29]. Comparison of the isotherms for the monolayers of SP-B, SP-C and SP-B/SP-C (Fig. 1) with those previously reported from this laboratory [17,32,33] revealed that the area per amino acid residue at a given surface pressure was within the limits of variability reported before [17]. In the present study, however, maximal surface pressures of about 40–45 mN/m were attained in the films of the proteins compared to about 30–35 mN/m, reported before [17,32,33]. The difference, as discussed in detail elsewhere [17], arose from the use of a Langmuir trough of a specific design in our previous study, which yielded somewhat low values in the region of high surface pressures.

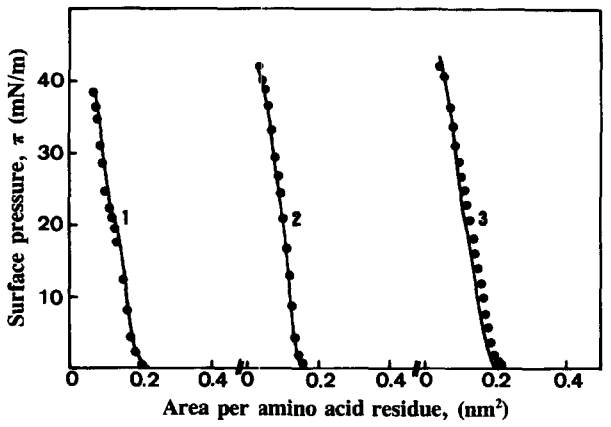

Fig. 1. Surface pressure versus mean area per amino acid residue for spread monolayers of SP-B (1), SP-C (2) and SP-B/SP-C (2:1, w/w) (3). The subphase was 150 mM NaCl plus 2 mM $CaCl_2$ (continuous lines) or 150 mM NaCl (closed circles).

The isotherms for the pure phospholipid and phospholipid-protein films were essentially independent of the trough design. In separate experiments we examined the behavior of some of the protein/DPPG monolayers in the presence and in the absence of calcium using the trough with the continuous teflon ribbon barrier. These measurements confirmed the result of Ca^{2+}-dependent behavior of the protein/DPPG films described below.

3.2. Monolayers of surfactant protein SP-B and phospholipids

Mixed films of SP-B and DPPC

Surface pressure (π)-area measurements in the presence of calcium in the subphase were performed on monolayers of DPPC alone and DPPC plus SP-B (data not shown). The bivalent ions had no effect on the isotherm of the zwitterionic DPPC. Ca^{2+} binds weakly to the lecithin head group [34] and does not affect the surface pressure-area properties of monolayers of phosphatidylcholines [35]. The results from the measurements on the SP-B/DPPC monolayers in the presence of Ca^{2+} were very similar to those in the absence of calcium [17]. Similar to our previous observations [17], the isotherms for the mixed films containing a 'residue' fraction of SP-B of $X_r \geq 0.42$, or 10 weight% protein, displayed an inflection, essentially independent of the initial film composition, at $\pi \approx 45$ mN/m. At this pressure, which was about 5 mN/m higher than the collapse pressure of SP-B alone, expulsion of predominantly SP-B, the component with lower collapse pressure, likely commenced. Previously, we calculated that in the absence of calcium nearly pure SP-B (dimer) associated with only 1–2 molecules of DPPC was squeezed out at $\pi \geq 45$ mN/m from the SP-B/DPPC monolayers of $X_r \geq 0.45$, or 10 weight% [17]. This amount of DPPC was within the accuracy of determination of the composition of the excluded phases [17].

To characterize the interaction between the components in the SP-B/DPPC films, the mean area per 'residue', A_{mean}, at a given surface pressure was plotted versus the

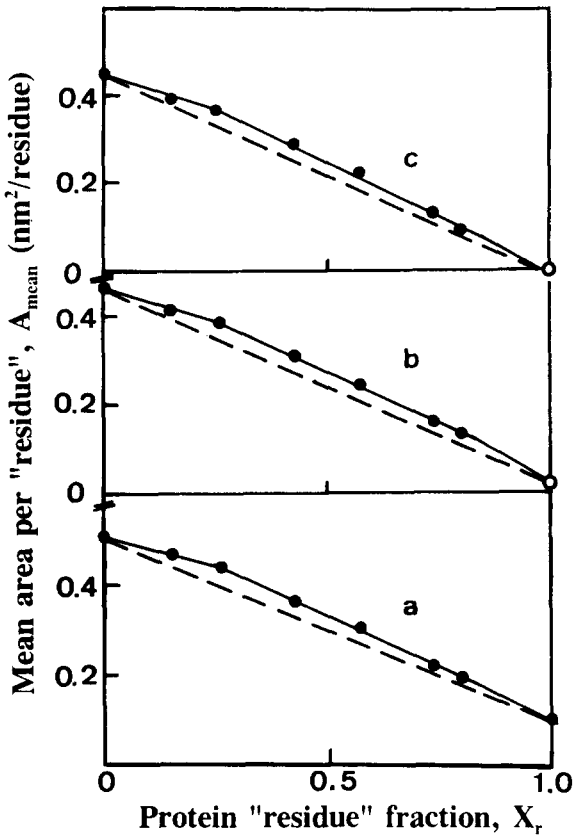

Fig. 2. Mean area per 'residue' versus initial protein concentration for SP-B/DPPC films at constant surface pressure π, π in mN/m: 25 (a), 45 (b), 55 (c). The open circles represent the value of A_{mean} extrapolated at the given surface pressure.

initial composition of the monolayers expressed by the fraction of amino acid residues of SP-B, X_r, (Fig. 2). The deviation in the mean areas in the binary monolayers (closed circles in Fig. 2), from the additivity rule (broken lines in Fig. 2) provided evidence for partial miscibility in the films. This property was like that observed in the absence of calcium and the deviations from ideal mixing of the films were similar to those seen in the absence of Ca^{2+} [17].

Fig. 2c shows that at $\pi = 55$ mN/m, a surface pressure higher than the exclusion pressure of the protein from the SP-B/DPPC films, the mean areas in the mixed monolayers were higher than those corresponding to ideal mixing for all lipid-protein monolayers studied, including those where squeeze-out was inferred ($X_r \geq 0.42$). We note that at $\pi = 45$ mN/m and $\pi = 55$ mN/m the values for the area per amino acid residue in the monolayers of pure SP-B, corresponding to $X_r = 1$ in Fig. 2b and c, were determined by graphical extrapolation of the isotherm for SP-B (curve 1, Fig. 1). The additive line in Fig. 2c, which passes through zero at $X_r = 1$, represents the area which a lipid molecule would occupy in the mixed films of various compositions following the complete exclusion of the protein from the surface; this area is assumed to be the same as that in the films of DPPC alone at the same surface pressure. The positive deviations of the experimental mean areas in the mixed films from the additive line in Fig. 2c are consistent with some protein being present in the films that were compressed to $\pi = 55$ mN/m, a pressure which was higher than the exclusion pressure of SP-B from the SP-B/DPPC films of composition $X_r \geq 0.42$ or 10 weight% protein. The observation of positive deviations in the $A_{mean}(X_r)$ plots in the whole range of protein concentrations studied suggested that though exclusion of some SP-B occurred at $\pi \geq 45$ mN/m during compression of the SP-B/DPPC films, part of the initially-spread protein remained in the lipid-enriched monolayers at the higher pressures. This might be clarified by dividing the trough area only by the number of DPPC molecules initially spread in the SP-B/DPPC films, and by comparing the isotherms thus obtained with the isotherm for the monolayers of pure DPPC (Fig. 3). The displacement of the isotherms for the lipid-protein films (curves 2 and 3 in Fig. 3) toward high areas compared to the isotherm for DPPC alone (curve 1) indicated, that independently of the initial concentration of SP-B in the films, some SP-B was present in the monolayers in the whole range of surface pressures measured. Apparently, for the protein-rich films, where squeeze-out of SP-B was seen (curve 3), only part of the initially-spread protein was excluded at $\pi \geq 45$ mN/m. The results in Fig. 3, curve 3, also suggested that DPPC, the component with higher collapse pressure, was not removed by SP-B during squeeze-out at $\pi \approx 45$ mN/m. If an appreciable amount of DPPC had been excluded from the films along with the protein, then the isotherm for the

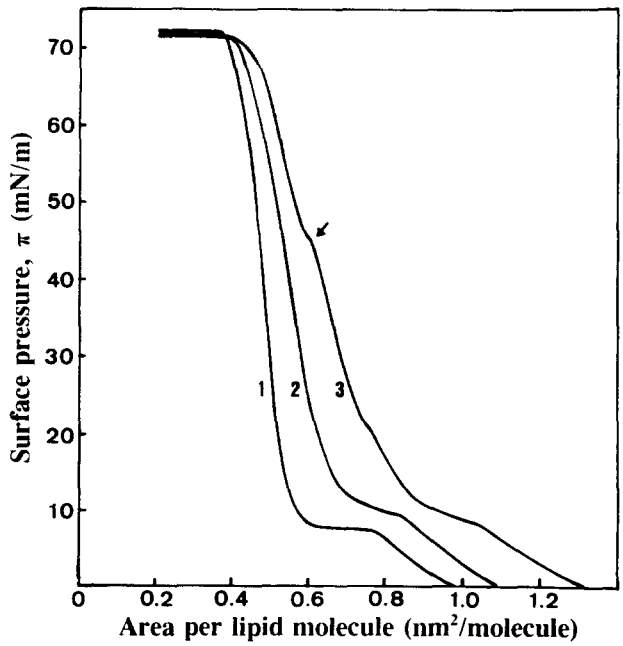

Fig. 3. Surface pressure as a function of area per lipid molecule for monolayers of DPPC (1), DPPC plus 5 weight% SP-B, or $X_r = 0.26$ (2) and DPPC plus 17 weight% SP-B, or $X_r = 0.58$ (3).

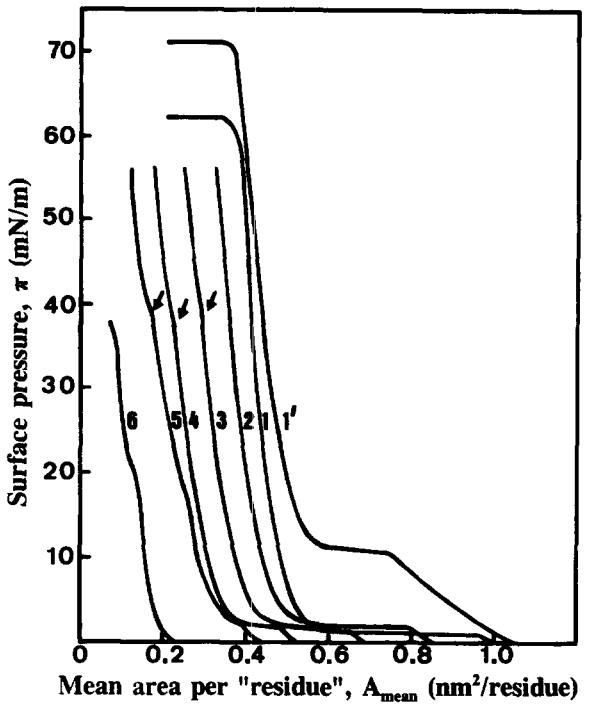

Fig. 4. Surface pressure-area curves for SP-B/DPPG films of initial protein concentration, X_r: 0.0 (1), 0.26 (2), 0.43 (3), 0.61 (4), 0.74 (5), 1.0 (6). The subphase was 150 mM NaCl plus 2 mM $CaCl_2$. Curve 1' represents the isotherm for $X_r = 0.0$ in the absence of calcium.

SP-B/DPPC monolayer of $X_r = 0.58$, curve 3 in Fig. 3, would have been shifted to lower areas compared to the isotherm for DPPC alone.

Mixed films of SP-B with DPPG

Fig. 4 shows the results from measurements on films of SP-B, DPPG and their mixtures in the presence of calcium. Curve 1' represents the isotherm for DPPG in the absence of Ca^{2+} in the subphase. Above pH 4, for subphase concentrations of NaCl above 10 mM, DPPG forms fully ionized monolayers [36]. Therefore, in the absence of Ca^{2+} and at physiological pH direct electrostatic interactions between the basic SP-B and DPPG may occur. Our previous measurements on SP-B/DPPG monolayers in the absence of Ca^{2+} suggested that non-polar interactions determined the properties of the mixed films [17].

The pressure-area isotherm for DPPG films measured in the presence of calcium in the subphase (curve 1 in Fig. 4) demonstrates the effect of the bivalent ions on the phase properties, molecular packing and collapse pressure of the acidic DPPG. At surface pressures corresponding to the condensed state of the films of DPPG, curve 1 was superimposable with those obtained by others for DPPG on monovalent salt subphases at pH 2 or on 10 mM $CaCl_2$, which were regarded as the most condensed forms of films of this lipid [37,38]. The effect of Ca^{2+} on the monolayer characteristics of DPPG at the air/water interface was consistent with the ability of Ca^{2+} to bind to the phosphatidylglycerol head group. Neutralization of the charges of acidic phospholipids by calcium causes a reduction in the electrostatic repulsion between adjacent molecules, and hence an increase in the lateral packing density of the monolayers [34,39,40]. Ca^{2+}-binding can also induce changes in the hydration state of the phospholipid polar head groups [41].

The $\pi(A_{mean})$ plots for SP-B/DPPG films which contained more than 10 weight% SP-B, or $X_r \geq 0.42$, displayed a characteristic inflection point at $\pi \approx 40$ mN/m (Fig. 4, curves 3-5). This pressure corresponded to the collapse pressure of SP-B and this suggested that possibly SP-B was squeezed out from the films. It is important to note, that under the same experimental conditions (rate of compression, trough design, temperature, subphase), in the absence of calcium in the subphase, we determined an exclusion pressure of about 45 mN/m for SP-B in the films with DPPG (data not shown). The decrease in the exclusion pressure of SP-B from the SP-B/DPPG films in the presence of Ca^{2+}, compared with the exclusion pressure in the absence of Ca^{2+}, is consistent with diminished interactions between the protein and phospholipid. The interfacial behavior of a lipid-protein monolayer is determined by van der Waals interactions between the hydrophobic parts of the molecules, electrostatic forces between the head groups of the components, and geometric perturbations in the mixed films [42]. Through its effect on the ionization state of DPPG, Ca^{2+} possibly screened putative electrostatic interactions between the basic SP-B and DPPG. As a result, a decrease in the overall interaction between the protein and DPPG was observed.

On the other hand, the exclusion pressure for SP-B from the DPPG-Ca films ($\pi \approx 40$ mN/m, Fig. 4) was lower than the exclusion pressure of the protein from the DPPC films in the presence of calcium ($\pi \approx 45$ mN/m, Fig. 3, curve 3). Assuming that in the presence of calcium, both DPPC and DPPG formed neutral films, then SP-B appeared to interact to a lesser extent with the phospholipid of a denser molecular packing, namely DPPG-Ca. For example, at $\pi = 40$ mN/m in the presence of Ca^{2+} we determined 0.41 nm^2/molecule for DPPG, compared to 0.46 ± 0.01 nm^2/molecule (mean ± S.D., $n = 4$) for DPPC monolayers. Therefore it seems that Ca^{2+} modified the interactions between SP-B and DPPG through its effects on both the ionization state and the intermolecular packing of the phospholipid at the air/water interface.

The dependence of the mean areas per 'residue' in the SP-B/DPPG films on the initial concentration of protein, in the presence of calcium (Fig. 5), was similar to that observed in the absence of Ca^{2+} [17]. The $A_{mean}(X_r)$ plots for the mixed films of SP-B with either DPPC (Fig. 2) or DPPG (Fig. 5) show similar behavior, regardless of the chemical nature of the polar head group. This suggested that interactions between the acyl chains of the phospholipids with the hydrophobic parts of SP-B molecule determined the deviation of the SP-B/lipid films from ideal

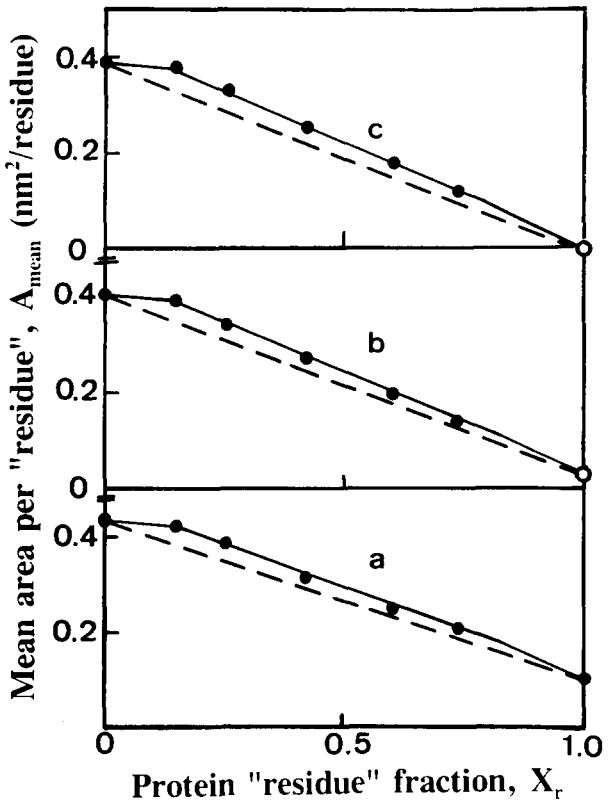

Fig. 5. Mean area per 'residue' versus initial protein concentration for SP-B/DPPG films at constant surface pressure, π, mN/m: 25 (a), 45 (b), 55 (c). The open circles represent the extrapolated value of A_{mean} at the given surface pressure.

behavior. At surface pressures higher than the exclusion pressure for SP-B from the SP-B/DPPG-Ca films the $A_{mean}(X_r)$ diagrams showed positive deviations from additivity for all compositions studied, including the binary mixtures where squeeze-out was observed (Fig. 5c). This property of the $A_{mean}(X_r)$ plots for SP-B/DPPG monolayers, similar to that for SP-B/DPPC films, suggested that SP-B was partially excluded during compression of the SP-B/DPPG films of $X_r \geq 0.43$ and the exclusion of the protein was not accompanied by removal of phospholipid. Such a low capacity of SP-B to remove phospholipid from the SP-B/DPPG films was also observed in the absence of calcium [17].

3.3. Monolayers of surfactant protein SP-C and phospholipids

Mixed films of SP-C and DPPC

The $\pi(A_{mean})$ curves for the SP-C/DPPC films in the presence of Ca^{2+} (data not shown) resembled those obtained in the absence of calcium [32]. The isotherms for the SP-C/DPPC films, which contained more than 5 weight% protein ($X_r \geq 0.25$), displayed a plateau-like region at $\pi = 51–53$ mN/m. As we suggested before, in the absence of calcium, at this pressure, squeeze-out of SP-C from the SP-C/DPPC films likely occurred and it was accompanied by removal of phospholipid [32].

In Fig. 6 the data from surface pressure-area measurements on SP-C/DPPC films in the presence of Ca^{2+} were plotted as mean area per 'residue' in the films versus their initial composition. The diagrams in Fig. 6 were very similar to those constructed in the absence of calcium [32]. The negative deviations in the mean areas per 'residue' from the additive line, for $X_r \geq 0.30$ at surface pressures higher than the exclusion pressure of SP-C are consistent with removal of DPPC molecules together with the protein (Fig. 6c). This result can be better understood by comparison of the isotherms for the SP-C/DPPC films, recalculated on an area per lipid molecule scale, with the isotherm for the monolayers of DPPC alone (Fig. 7). Curve 2 in Fig. 7, which represents the result for SP-C/DPPC monolayers which did not exhibit squeeze-out plateau ($X_r = 0.14$, or 2.5 weight%, SP-C), was displaced to higher areas than the isotherm for DPPC alone (curve 1, Fig. 7). At the lower concentrations of protein, it appears that the whole amount of the initially-spread SP-C remained in the mixed films during compression to $\pi \approx 72$ mN/m. For SP-C/DPPC monolayers which contained higher amounts of protein, e.g., $X_r = 0.41$ or 10 weight%, at surface pressures above the exclusion pressure of the protein, DPPC apparently

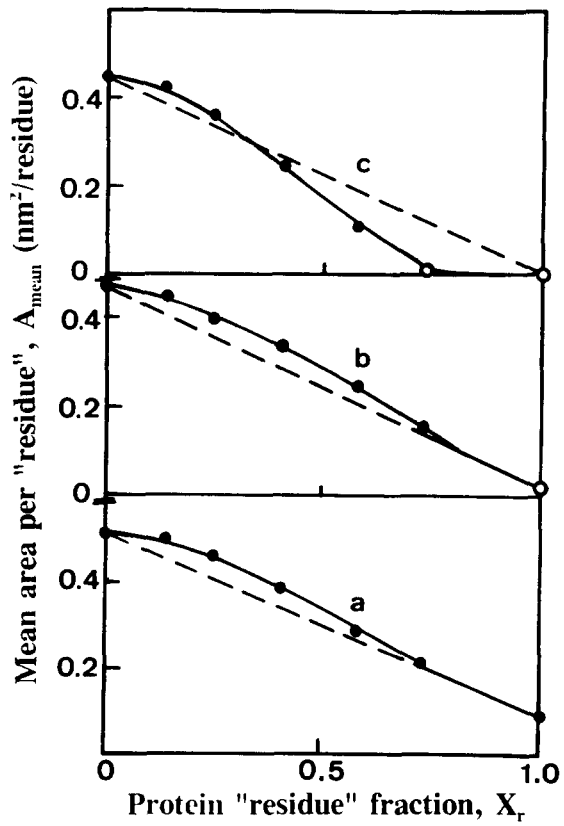

Fig. 6. Mean area per 'residue' in the SP-C/DPPC films as a function of the initial composition at constant surface pressure, π in mN/m: 25 (a), 45 (b), 55 (c). The open circles represent the extrapolated value of A_{mean} at the given surface pressure.

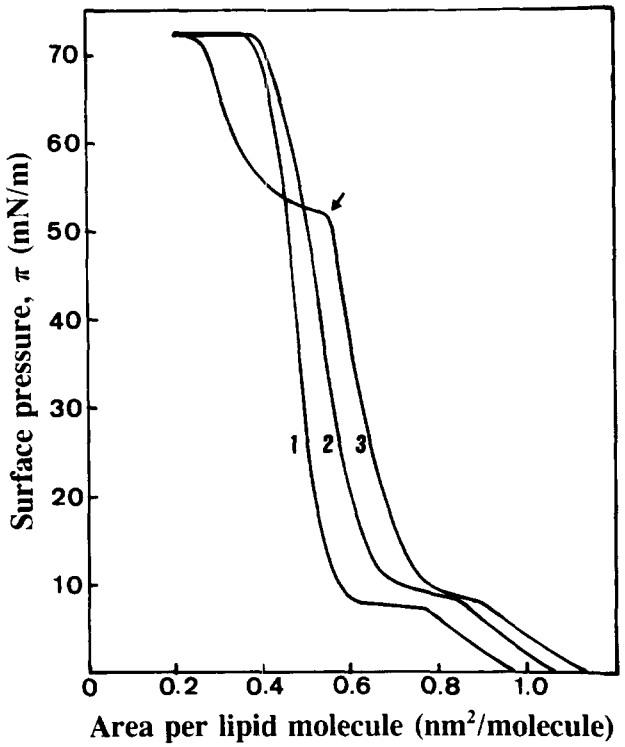

Fig. 7. Surface pressure versus area per lipid molecule for monolayers of DPPC (1), DPPC plus 2.5 weight% SP-C, or $X_r = 0.14$ (2) and DPPC plus 10 weight% SP-C, or $X_r = 0.41$ (3).

occupied a smaller area in the mixed films than it did in the films of pure phospholipid (compare curves 3 and 1 in Fig. 7). The observation was consistent with partial loss of phospholipid molecules along with the protein at surface pressures higher than approx. 55 mN/m. Therefore, contrary to the observation for the mixed films of SP-B plus DPPC (Fig. 3), SP-C was capable of removing DPPC from the SP-C/DPPC monolayers at surface pressures well below the collapse pressure of DPPC. Our previous calculations of the compositions of the phases lost from the SP-C/DPPC films at $\pi = 55$ mN/m showed that, in the absence of calcium, about 7-8 molecules of DPPC per mol of SP-C were removed from the films [32]. Similar calculations showed, that in the presence of calcium, about 12 molecules of DPPC were removed together with SP-C from the SP-C/DPPC films of initial compositions $X_r = 0.41$ and $X_r = 0.58$ at $\pi = 55$ mN/m. The numbers of phospholipids removed by SP-C from the SP-C/DPPC films in the two studies can not be compared directly because, in addition to the presence of calcium, other experimental conditions varied slightly between the two series of experiments. However, the results in the two studies clearly demonstrate that, in the presence or absence of Ca^{2+}, SP-C is capable of removing DPPC from the mixed SP-C/DPPC films.

Mixed films of SP-C and DPPG

The surface pressure-area isotherms for the films composed of SP-C plus DPPG are shown in Fig. 8. The lipid-protein films which contained more than 5 weight% SP-C, or $X_r \geq 0.25$ (Fig. 8, curves 4–7) displayed kink points at about 45 mN/m. At this pressure, corresponding to the collapse pressure of the films of pure SP-C, possibly SP-C was excluded from the films. The exclusion pressure of SP-C from SP-C/DPPG films in the presence of Ca^{2+} was lower than that determined in the absence of Ca^{2+} (about 50 mN/m [32]).

The plots of the mean area per 'residue' in the SP-C/DPPG films versus their initial composition at surface pressures below the exclusion pressure for SP-C, (Fig. 9a), showed features similar to those seen in the absence of calcium [32]. At surface pressures above the exclusion pressure for SP-C, however, Ca^{2+} modified the characteristics of the $A_{mean}(X_r)$ diagrams at the high protein concentrations. In the absence of Ca^{2+}, at $\pi = 55$ mN/m, the $A_{mean}(X_r)$ plots for the SP-C/DPPG films of compositions $X_r > 0.30$ displayed negative deviations from the additive rule [32], very similar to those seen for the SP-C/DPPC films in Fig. 6c. This observation was consistent with removal of phospholipid during exclusion of SP-C. Calculations of the compositions of the phases excluded at $\pi = 55$ mN/m showed that in the absence of Ca^{2+} about 11 molecules of DPPG were taken away from the surface during squeeze-out of a mol of SP-C, i.e., there was potential for SP-C to remove DPPG during compression of the films [32].

In the presence of calcium, at $\pi = 55$ mN/m, the mean area per 'residue' showed positive deviations from the additivity rule for all films of initial protein concentrations

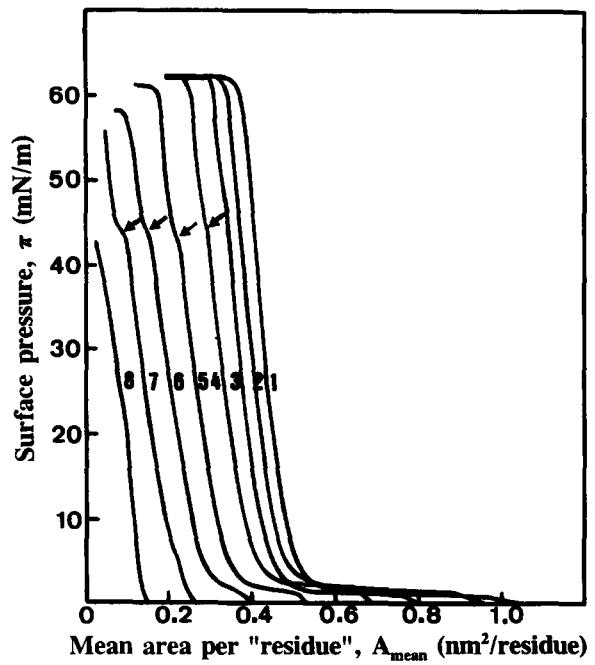

Fig. 8. Surface pressure versus mean area per 'residue' for SP-C/DPPG films of initial composition, X_r: 0.0 (1), 0.14 (2), 0.25 (3), 0.41 (4), 0.58 (5), 0.73 (6), 0.86 (7), 1.0 (8). The subphase was 150 mM NaCl plus 2 mM $CaCl_2$.

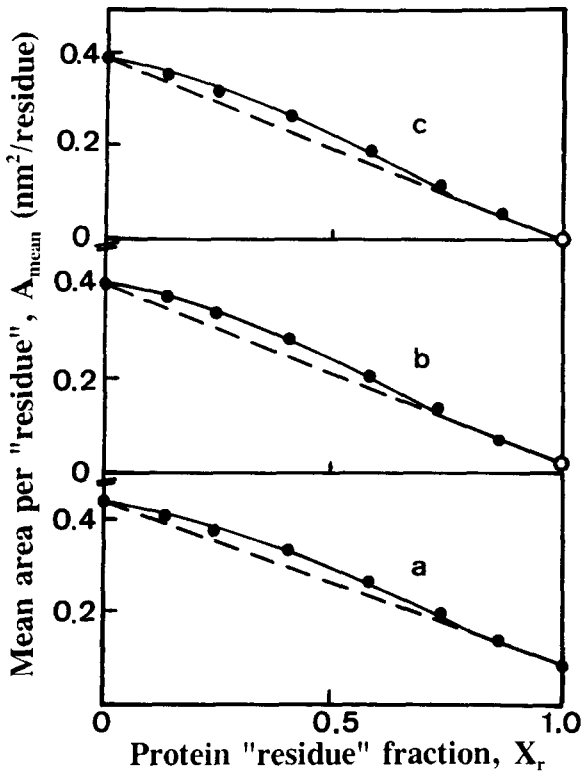

Fig. 9. Mean area per 'residue' in the SP-C/DPPG films as a function of initial film composition at constant surface pressure, π, mN/m: 25 (a), 45 (b), 55 (c). The open circles represent the extrapolated value of A_{mean} at the given surface pressure.

$X_r \leq 0.73$, or 30 weight%, i.e., including the films where squeeze-out was seen (Fig. 9c). As discussed earlier in this study, such a behavior of the $A_{mean}(X_r)$ plots at surface pressures above the exclusion pressure of the protein from the lipid/protein films is consistent with some protein remaining in the films after its partial squeeze-out. The absence of negative deviations from additivity in the $A_{mean}(X_r)$ plots in Fig. 9c, as opposed to the observations in the absence of calcium [32], also suggested that Ca^{2+} modified the interactions between the lipid and protein so that the exclusion of SP-C was not accompanied by removal of DPPG. Comparison of the plot of surface pressure versus area per lipid molecule for SP-C/DPPG monolayers of $X_r = 0.41$, or 10 weight%, (curve 2 in Fig. 10) with that for the films of DPPG alone (curve 1 in Fig. 10), indicated that SP-C was partially ejected from the mixed films at $\pi \geq 45$ mN/m and it did not remove phospholipid during exclusion.

It is worth noting that in the presence of calcium the exclusion pressure of SP-C from the mixed films with the zwitterionic DPPC (about 50 mN/m, Fig. 7, curve 3) was higher than the exclusion pressure from the DPPG-Ca films (about 45 mN/m, Fig. 10, curve 2). SP-C was capable of removing phospholipid during squeeze-out from SP-C/DPPC films independently of the presence of calcium, whereas in the films of SP-C with DPPG-Ca the protein did not remove DPPG during squeeze-out. These differences in the interactions of SP-C with DPPC and DPPG, which both presumably form neutral films in the presence of Ca^{2+}, suggested that the interactions of the protein with the phospholipids were affected by differences in the molecular packing of the lipids at the air/water interface. As discussed above, in the presence of Ca^{2+} the films of DPPG showed higher packing density relative to the films of DPPC.

Similar to the effect of Ca^{2+} on the interactions of SP-B with DPPG, the influence of the bivalent ion on the association of the basic SP-C with the acidic DPPG could be interpreted in terms of changes in the ionization state and molecular packing of DPPG caused by the calcium in the subphase. Surface pressure measurements alone are insufficient to distinguish between the contributions of the electrostatic and van der Waals forces to such an effect of Ca^{2+} on the properties of the lipid-protein films.

3.4. Monolayers of surfactant proteins SP-B plus SP-C with phospholipids

Ternary monolayers of SP-B / SP-C plus DPPC

The surface pressure-area plots for films composed of SP-B/SP-C plus DPPC in the presence of calcium in the subphase (data not shown) were very similar to those measured in the absence of Ca^{2+} [33]. The (SP-B/SP-C)/DPPC films which contained $\geq$ 17 weight% protein, or $X_r \geq 0.57$ displayed two kinks in the region of high

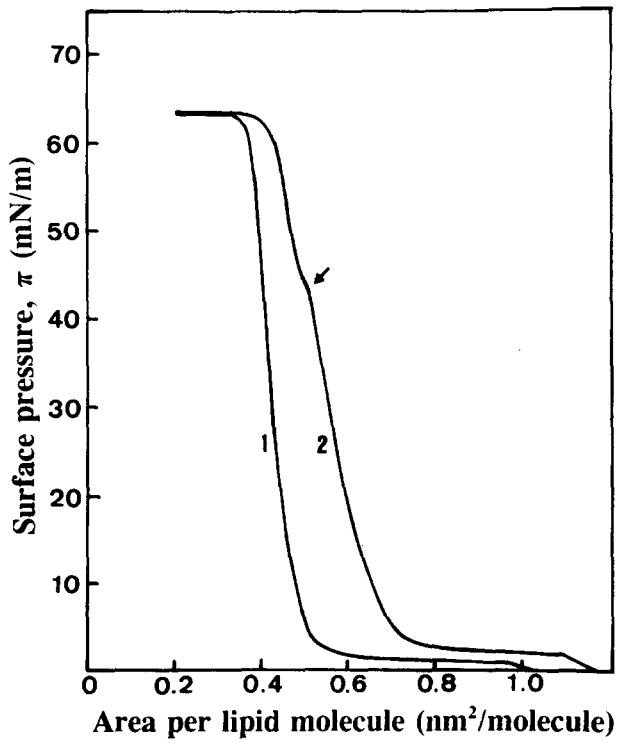

Fig. 10. Surface pressure versus area per lipid molecule for monolayers of DPPG (1) and DPPG plus 10 weight% SP-C, or $X_r = 0.41$ (2).

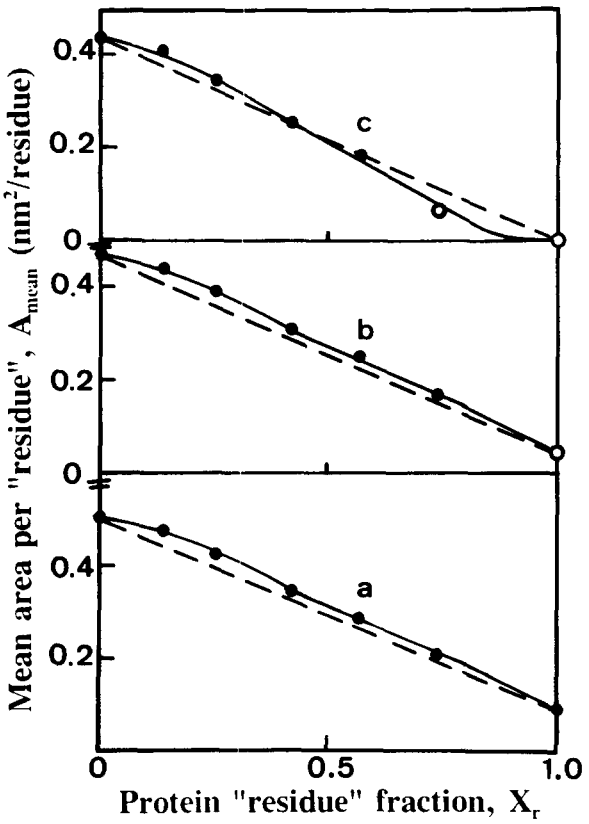

Fig. 11. Mean area per 'residue' in the (SP-B/SP-C)/DPPC films versus monolayer composition at constant surface pressure, π, mN/m: 25 (a), 45 (b), 60 (c). The open circles represent extrapolated value of A_{mean} at the given surface pressure.

surface pressures, preceding the collapse at about 72 mN/m (data not shown). The kink at about 45 mN/m corresponded to the exclusion pressure of SP-B from the SP-B/DPPC films, whereas the inflection at about 50 mN/m coincided with the squeeze-out pressure of SP-C from SP-C/DPPC films. These observations suggested that SP-B and SP-C did not interact with one another in the ternary monolayers with DPPC and they were separately squeeze-out during compression of the films.

The plots of the mean area per 'residue' versus monolayer composition, shown in Fig. 11, were similar to those in the absence of calcium [33]. For pressures lower than the exclusion pressures for SP-B and SP-C ($\pi < 45$ mN/m) the 'expansion' in the ternary films was a sum of the 'expansions' in the two binary SP-B/DPPC and SP-C/DPPC films of the same composition and surface pressure (results are not shown). This observation was consistent with independent binary SP-B/DPPC and SP-C/DPPC interactions in the three-component monolayers, as were seen in the absence of Ca^{2+} [33]. The negative deviations in the mean area per 'residue' for the protein-rich mixed films at $\pi > 55$ mN/m (Fig. 11c) suggested that DPPC was removed from the ternary films during squeeze-out of the proteins. The removal of DPPC from the ternary films was likely due to the presence of SP-C in the films.

Ternary monolayers of SP-B / SP-C plus DPPG

In the presence of Ca^{2+}, the $\pi(A_{mean})$ curves for the (SP-B/SP-C)/DPPG films of initial compositions $X_r \geq 0.42$, or 10 weight% protein, displayed one kink point at $\pi \approx 40$ mN/m, preceding the collapse at about 65 mN/m (Fig. 12). This observation suggested a combined exclusion of SP-B and SP-C from the ternary films at a surface pressure comparable with the exclusion pressures of SP-B and SP-C from their binary films with DPPG (Figs. 4 and 8). Combined squeeze-out of the two proteins from the (SP-B/SP-C)/DPPG films was also seen in the absence of calcium [33]. In that case, however, the exclusion pressure for the two proteins was about 50 mN/m. Therefore, the divalent ions acted to decrease the exclusion pressure of the proteins from the ternary films with DPPG compared to their exclusion pressure in the absence of Ca^{2+}.

Calcium ions in the subphase also modified the dependence of the mean area per 'residue' in the (SP-B/SP-C)/DPPG films on monolayer composition (Fig. 13). In the presence of Ca^{2+}, at the low surface pressures, the 'expansion' in the ternary (SP-B/SP-C)/DPPG monolayers was smaller compared with the effects seen in the binary monolayers of either SP-B or SP-C plus DPPG (Figs. 5 and 9). Contrary to this observation, in the absence of Ca^{2+}, SP-B and SP-C were found to have synergistic effects in perturbing the monolayer packing of DPPG [33]. The $A_{mean}(X_r)$ diagrams for the (SP-B/SP-C)/DPPG monolayers at high surface pressures, e.g., $\pi = 60$ mN/m,

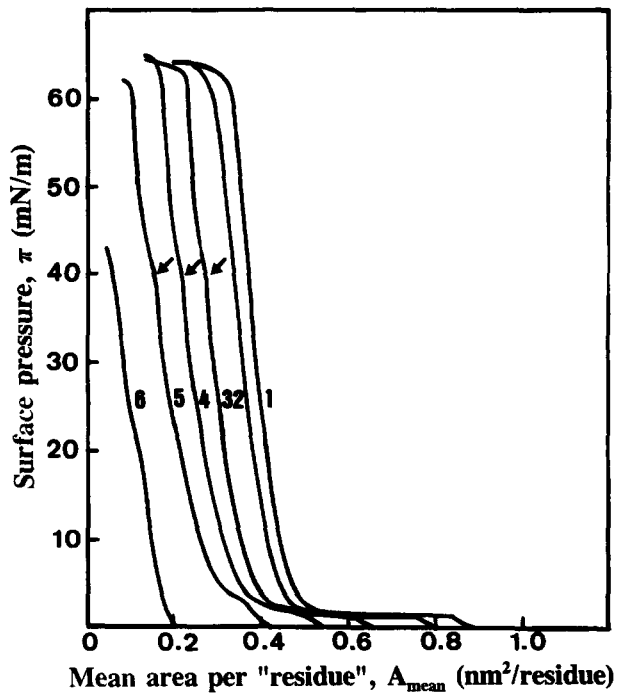

Fig. 12. Surface pressure versus mean area per 'residue' for mixed monolayers of SP-B/SP-C (2:1, w/w) with DPPG. The initial composition of the films, X_r: 0.15 (1), 0.25 (2), 0.42 (3), 0.57 (4), 0.74 (5), 1.0 (6). The subphase was 150 mM NaCl plus 2 mM $CaCl_2$.

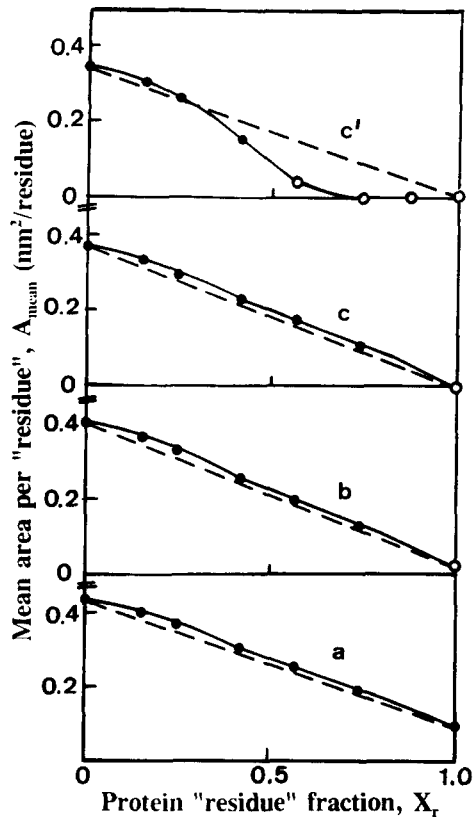

Fig. 13. Mean area per 'residue' in the (SP-B/SP-C)/DPPG films versus initial monolayer composition at constant surface pressure, π, mN/m: 25 (a), 45 (b), 60 (c). $A_{mean}(X_r)$ at $\pi = 60$ mN/m in the absence of Ca^{2+} (c') [33]. The open circles represent extrapolated value of A_{mean} at the given surface pressure.

displayed considerably different behavior depending on the presence of Ca^{2+} in the subphase. In the presence of Ca^{2+}, $A_{mean}(X_r)$ plots in Fig. 13c did not show negative deviations from the additive rule at surface pressures above the exclusion pressures for the proteins, contrary to the observations in the absence of calcium (Fig. 13c'). As discussed earlier in this study, negative deviations from additive behavior of the $A_{mean}(X_r)$ plots at surface pressures above the exclusion pressure of the protein from the mixed films are consistent with removal of phospholipid during squeeze-out of the protein. Accordingly, our previous calculations showed, that in the absence of Ca^{2+}, for (SP-B/SP-C)/DPPG films of initial composition $X_r = 0.74$, corresponding to 30 weight% protein, about 27 mol of DPPG were removed from the ternary films during squeeze-out of a mol of SP-B/SP-C at $\pi = 55$ mN/m [33]. Contrary to these results, the $A_{mean}(X_r)$ plots for (SP-B/SP-C)/DPPG films in the presence of calcium, shown in Fig. 13c, suggested that lipid was not removed during squeeze-out of the proteins. Therefore, it appears that the overall effect of Ca^{2+} on the films of SP-B/SP-C plus DPPG was to weaken both the interactions between the two proteins in the ternary films, and the interactions of the two proteins with DPPG. Though there was no direct evidence for a formation of a specific complex between the two proteins and the acidic DPPG, the surface pressure measurements in the absence of calcium, gave indication for enhanced interactions of the combination SP-B/SP-C with DPPG, compared to the interactions of each individual protein with DPPG [33]. Calcium ions in the subphase, possibly through their action on DPPG, eliminated the interdependent effect of SP-B and SP-C on DPPG films which was seen in the absence of calcium [33].

4. Conclusions

The pressure-area characteristics of the spread films of the hydrophobic surfactant proteins SP-B, SP-C and SP-B/SP-C (2:1, w/w) were independent of the presence of calcium in the subphase. Ca^{2+} did not influence the properties of the mixed films of either SP-B, or SP-C or SP-B/SP-C with the zwitterionic DPPC.

Calcium ions had effect on the behavior of the monolayers of the surfactant proteins with DPPG. This led to: (i) decrease in the exclusion pressure of the proteins from the mixed films with DPPG in comparison with their exclusion pressures in the absence of calcium (for SP-B/DPPG, SP-C/DPPG and (SP-B/SP-C)/DPPG films); (ii) suppression of the ability of the proteins to remove DPPG from the films in comparison with the effects seen in the absence of calcium (in SP-C/DPPG and (SP-B/SP-C)/DPPG films). Likewise, Ca^{2+} influenced the interactions between SP-B and SP-C in the ternary films with DPPG and this resulted in an additive behavior of the proteins, different from their synergistic action in the absence of calcium [33].

The experimental findings that calcium weakened the interactions between the hydrophobic surfactant proteins SP-B and SP-C, and their combination SP-B/SP-C, with the acidic DPPG could be interpreted in terms of neutralization of the negative charges of DPPG by Ca^{2+} [34,39,40]. Binding between calcium and DPPG and formation of a neutral species, DPPG-Ca, which did not interact with the proteins through putative electrostatic forces could account for the reduction in the overall interactions between the basic proteins and the phospholipid in the presence of calcium. On the other hand, through its effect on the molecular packing of the lipid hydrocarbon chains, calcium possibly also affected the hydrophobic interactions between DPPG and the proteins. From the results in this and previous studies [17,32,33] it could be concluded that calcium ions modified the observed interfacial properties of the mixed films of the hydrophobic surfactant proteins with DPPG. This observation is consistent with results from research on model bilayer systems, which have shown that Ca^{2+} accelerated surface adsorption of lipid dispersions containing hydrophobic surfactant protein only in the presence of acidic phospholipids [10–

12]. The mechanism for the effect of Ca^{2+} on the lipid–protein interactions in the monolayers of the hydrophobic surfactant proteins with DPPG possibly involved binding of the bivalent cations to the phospholipid head groups which led to changes in the ionization state and intermolecular packing of DPPG at the air/water interface.

Acknowledgements

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