Effect of Surfactant Protein A on the Physical Properties and Surface Activity of KL_4-Surfactant

Alejandra Sáenz,* Olga Cañadas,* Luís A. Bagatolli,† Fernando Sánchez-Barbero,* Mark E. Johnson,‡ and Cristina Casals*

*Department of Biochemistry and Molecular Biology I, Complutense University of Madrid, Madrid, Spain; †MEMPHYS-Center for Biomembrane Physics, Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark; and ‡Discovery Laboratories, Mountain View, California

ABSTRACT SP-A, the major protein component of pulmonary surfactant, is absent in exogenous surfactants currently used in clinical practice. However, it is thought that therapeutic properties of natural surfactants improve after enrichment with SP-A. The objective of this study was to determine SP-A effects on physical properties and surface activity of a new synthetic lung surfactant based on a cationic and hydrophobic 21-residue peptide KLLLLKLLLLKLLLLKLLLLK, KL_4. We have analyzed the interaction of SP-A with liposomes consisting of DPPC/POPG/PA (28:9:5.6, w/w/w) with and without 0.57 mol % KL_4 peptide. We found that SP-A had a concentration-dependent effect on the surface activity of KL_4-DPPC/POPG/PA membranes but not on that of an animal-derived LES. The surface activity of KL_4-surfactant significantly improved after enrichment with 2.5–5 wt % SP-A. However, it worsened at SP-A concentrations $\geq$10 wt %. This was due to the fluidizing effect of supraphysiological SP-A concentrations on KL_4-DPPC/POPG/PA membranes as determined by fluorescence anisotropy measurements, calorimetric studies, and confocal fluorescence microscopy of GUVs. High SP-A concentrations caused disappearance of the solid/fluid phase coexistence of KL_4-surfactant, suggesting that phase coexistence might be important for the surface adsorption process.

INTRODUCTION

Pulmonary surfactant is a heterogeneous lipid-protein complex that overlies the alveolar epithelium and stabilizes the lung by reducing surface tension in the alveolus (1,2). This makes breathing easier and prevents alveolar edema. Surfactant is also involved in lung defense against inhaled pathogens and toxins and modulates the function of respiratory inflammatory cells (3). Lung immaturity and surfactant deficiency are the main factors in the pathogenesis of neonatal RDS. Surfactant dysfunction, caused by inactivation of surface active material in the airspaces, also contributes to respiratory failure in other forms of neonatal lung disease, such as meconium aspiration syndrome, and in ARDS (3–5).

Surfactant is composed of 90 wt % lipids and 10 wt % proteins. PLs are the major lipid component of surfactant, especially DPPC (1,2). PG represents a major unsaturated anionic component (1,2). Four surfactant proteins have been reported to exist in this material: the hydrophobic proteins SP-B and SP-C, which are inserted in surfactant membranes, and the collectins SP-A and SP-D. SP-B is a small hydrophobic protein that is essential for lung function and pulmonary homeostasis after birth. SP-B constitutes 0.7% of the total mass of isolated surfactant (1). Genetic absence of SP-B in both human beings and mice results in a lack of alveolar expansion and a lethal failure of pulmonary function (4). In contrast, genetic absence of SP-C, another small hydrophobic protein that constitutes 0.5 wt %, results in normal expansion of alveoli and pulmonary function (4). Both hydrophobic proteins enhance the spreading, adsorption, and stability of surfactant lipids required for the reduction of surface tension in the alveolus (1,2,4). SP-A and SP-D are large oligomeric proteins that belong to the collectin family involved in immune innate host defense (3). They are characterized by an N-terminal collagen-like domain and a globular C-terminal domain that includes a C-type CRD (3,6). SP-A comprises ~3–4% of the total mass of surfactant and is mainly associated with surfactant lipids (2,7). SP-D, on the other hand, constitutes ~0.5 wt % and is not associated with lipids (2). SP-A's ability to bind lipids 1), improves the adsorption and spreading of surfactant membranes onto an air-liquid interface (8,9); 2), protects surfactant biophysical activity from the inhibitory action of serum proteins (9); and 3), allows this protein to position and concentrate along with

Submitted May 29, 2006, and accepted for publication September 26, 2006.

Address reprint requests to Cristina Casals, Dept. of Biochemistry and Molecular Biology I, Faculty of Biology, Complutense University of Madrid, 28040 Madrid, Spain. Tel.: 34-91-3944261; Fax: 34-91-3944672; E-mail: ccasalsc@bio.ucm.es.

Abbreviations used: λ_m, emission wavelength (nm); λ_x, excitation wavelength (nm); ARDS, adult respiratory distress syndrome; BODIPY-PC, 2-(4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-*s*-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; Ch, cholesterol; CRD, carbohydrate recognition domain; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, 1,2-dipalmitoyl-phosphatidylcholine; DSC, differential scanning calorimetry; GUV, giant unilamellar vesicle; HPLC, high-performance liquid chromatography; LES, lipid extract surfactant; MLV, multilamellar vesicle; PA, palmitic acid; PG, phosphatidylglycerol; PL, phospholipid; POPG, 1-palmitoyl-2-oleoyl-phosphatidylglycerol; RDS, respiratory distress syndrome; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SP-A, surfactant protein A; SP-B, surfactant protein B; SP-C, surfactant protein C; SP-D, surfactant protein D; T_m, gel to fluid main phase transition temperature.

0006-3495/07/01/482/11 $2.00

doi: 10.1529/biophysj.106.090217

surfactant membranes at the front lines of defense against inhaled toxins or pathogens (3,6).

Airway instillation of surfactant is in general use for treatment of RDS in preterm babies (5). Replacement surfactants consist of lipid extract preparations obtained from animal bronchoalveolar fluids. Common components in these preparations are PLs, mainly DPPC, and the hydrophobic proteins SP-B and SP-C (5). Replacement surfactants do not contain SP-A. Lung function is apparently maintained with a surfactant devoid of SP-A at sufficient concentrations (10). However, several authors indicate that surfactant potency is impaired in the absence of SP-A (11,12). Bernhard and co-workers (12) concluded that the reduced SP-B/C content found in commercial replacement surfactants, together with the absence of SP-A, could be the reason for the diminished surface activity of replacement surfactants compared with native surfactants. This view is reinforced by the facts that addition of SP-A to a modified replacement surfactant (Survanta) enhances the therapeutic effect of this surfactant on dynamic and static lung compliance in ventilated premature newborn rabbits (13) and that it improves the physical and therapeutic properties of another animal-derived surfactant (Curosurf) (14).

Currently, many efforts are being made to develop synthetic surfactants since natural surfactants from animal sources involve microbiological, immunological, economic, and purity concerns. Synthetic surfactants consist of combinations of synthetic lipids and either synthetic or recombinant peptides (15). A synthetic lung surfactant formulation was developed based upon a cationic and hydrophobic 21-residue peptide (KL_4) (KLLLLKLLLLKLLLLKLLLLK, where ''K'' and ''L'' represent the amino acids lysine and leucine, respectively). KL_4 mimics the positive charge and hydrophobic residue distribution of SP-B (16). This synthetic surfactant is comprised of DPPC, POPG, PA, and KL_4 at weight ratios of 28:9.3:5.0:1.0, respectively. The KL_4 peptide concentration corresponds to 0.57 mol % and 2.3 wt %. At this concentration, KL_4 adopted a predominantly α-helical secondary structure in these membranes, independent of the presence of calcium (17), and is likely oriented with its backbone parallel to the interface. The surface activity of KL_4 peptide incorporated in surfactant-like bilayers and monolayers is well recognized (16–19). KL_4-surfactant (Surfaxin) has been found to be very effective in clinical trials of human RDS (20,21), in experimental and clinical meconium aspiration syndrome (22,23), and in patients with ARDS (24).

The effect of enrichment of KL_4-surfactant with SP-A on its biophysical activity is unknown. SP-A might interact with the cationic and hydrophobic peptide KL_4 inserted in the membrane surface given the negative charge in the SP-A surface (25) with pI varying between pH 4.5 and 5.2. The aims of this study were to determine SP-A effects on physical properties and lateral lipid organization of KL_4-containing surfactant-like membranes and to determine whether supplementation of KL_4-surfactant with SP-A results in improvement or impairment of its surface activity. Parallel experiments were performed with a porcine LES as a benchmark of natural surfactant. We found that SP-A had a concentration-dependent effect on the surface activity of KL_4-DPPC/POPG/PA membranes but not on that of LES, which contains all the lipid associated with surfactant plus the hydrophobic surfactant proteins SP-B and SP-C. Our results demonstrate that, at physiological concentrations, SP-A improved the efficacy of KL_4-surfactant. However, at SP-A supraphysiological concentrations, the surface activity of this synthetic surfactant diminished as a consequence of the fluidizing effect of SP-A on surfactant-like membranes with, but not without, KL_4.

MATERIALS AND METHODS

Materials

Synthetic lipids DPPC, POPG, and PA were purchased from Avanti Polar Lipids (Birmingham, AL). The organic solvents (methanol and chloroform) used to dissolve lipids were HPLC-grade (Scharlau, Barcelona, Spain). Bodipy-PC and DPH were purchased from Molecular Probes (Eugene, OR). All other reagents were of analytical grade and obtained from Merck (Darmstadt, Germany).

MLVs of DPPC/POPG/PA (28:9.4:5.1, w/w/w), containing different amounts of KL_4 peptide, were prepared as previously reported (17). The sample solutions were prepared by mixed stock solution of the lipid and the peptide, both prepared in chloroform/methanol, to achieve the desired lipid/peptide ratio.

Isolation of SP-A

SP-A was isolated from bronchoalveolar lavage of patients with alveolar proteinosis using a sequential butanol and octylglucoside extraction (8). The purity of SP-A was checked by one-dimensional SDS-PAGE in 12% acrylamide under reducing conditions and mass spectrometry. Quantification of SP-A was carried out by amino acid analysis in a Beckman System 6300 High Performance analyzer (Beckman Instruments, Palo Alto, CA). The oligomerization state of SP-A was assessed by electrophoresis under nondenaturing conditions and electron microscopy as reported elsewhere (26,27). SP-A isolated from alveolar proteinosis patients consisted of supratrimeric oligomers of at least 18 subunits. Each subunit had an apparent molecular weight of 36,000.

Isolation and analysis of pulmonary surfactant and LES

Pulmonary surfactant from porcine lungs was obtained as previously described (28,29). Briefly, cell-free bronchoalveolar lavage was centrifuged at $100{,}000 \times g$ for 2 h at 4°C to obtain the large surfactant aggregates in the resulting pellet. The separation of pulmonary surfactant from blood components was performed by NaBr density-gradient centrifugation at $116{,}000 \times g$ for 2 h at 4°C. Surfactant has a density of ~1.085 at 4°C, which is lower than that of most of the contaminating components of serum.

LES, which contains surfactant lipids, SP-B, and SP-C, was prepared by chloroform/methanol extraction of isolated surfactant (28). The organic solvent was then evaporated to dryness under a stream of nitrogen, and traces of solvent were subsequently removed by evacuation under reduced pressure overnight. MLVs of LES were prepared by hydrating the dry proteolipid film in buffer A containing 150 mM NaCl, 25 mM Hepes, pH

7.0, and allowing them to swell for 1 h at 45°C. After vortexing, the resulting MLVs were used for different assays.

Total PL was determined from aliquots of both native surfactant and LES by phosphorus analysis. PL classes were determined in the organic extract of porcine surfactant by TLC as in Casals et al. (28,29). Total surfactant Ch was determined enzymatically using the Sigma Diagnostic Cholesterol Kit. SP-B and SP-C content was determined in organic extracts of porcine surfactant using ELISA procedures described previously (30,31). For SP-B measurement, an anti-porcine SP-B antiserum was used. For SP-C measurement, an anti-recombinant human SP-C antiserum (generously donated by Altana Pharma, Konstanz, Germany) was used. There was cross-reactivity of this antiserum with porcine SP-C. The detection limit for porcine SP-C was 180 ± 2.5 ng/ml.

Adsorption assays

The ability of LES and DPPC/POPG/PA vesicles, with or without 0.57 mol % KL_4, to absorb onto and spread at the air-water interface was tested in the absence and presence of different amounts of SP-A in a Wilhelmy-like highly sensitive surface microbalance (17,26,27,29). The lipid mixtures were injected into the hypophase chamber of the Teflon dish, which contained 6 ml of buffer A either with or without 5 mM $CaCl_2$ in the presence and absence of SP-A. Interfacial adsorption was measured after the change in surface tension as a function of time. For each preparation, the analysis was repeated at least three times.

Emission anisotropy measurements

The required amounts of the stock solutions of DPPC/POPG/PA and KL_4 were mixed with DPH at a probe/PL molar ratio of 1:200 (final PL concentration of 1 mg/ml) as previously described (17,32). In cases where DPH was incorporated into LES, the probe dissolved in methanol was added to organic extracts of native surfactant at a DPH/PL molar ratio of 1:200 before solvent removal. DPH concentration was determined spectrophotometrically by absorbance at a wavelength of 350 nm, using a molar extinction coefficient in methanol of 88,000 M^{-1} cm^{-1}. LES and DPPC/POPG/PA vesicles, with or without KL_4, were prepared in buffer B (20 mM Tris-HCl, 130 mM NaCl, pH 7.6). Exposure to light was minimized throughout the vesicle preparation process. An appropriate amount of SP-A in buffer B was added to each aliquot of either LES, KL_4-DPPC/POPG/PA vesicles, or vesicles without KL_4 to give the desired final concentration of protein, with a <5% increase in volume for each aliquot.

Steady-state fluorescence emission anisotropy measurements were carried out using an SLM-Aminco AB-2 spectrofluorimeter equipped with Glam prism polarizers and a thermostated cuvette holder (±0.1°C) (Thermo Spectronic, Waltham, MA), using 5 × 5 mm path-length quartz cuvettes, as previously described (17,26,32). For each sample, fluorescence emission intensity data in parallel and perpendicular orientations with respect to the exciting beam were collected 10 times each and then averaged. Background intensities in DPH-free samples due to the vesicles and SP-A were subtracted from each recording of fluorescence intensity. λ_m and λ_x were set at 360 and 430 nm, respectively. Anisotropy, r, was calculated as

$$r = \frac{I_{\parallel} - G \cdot I_{\perp}}{I_{\parallel} + 2G \cdot I_{\perp}},$$

where $I_{\parallel}$ and $I_{\perp}$ are the parallel and perpendicular polarized intensities measured with the vertically polarized excitation light and G is the monochromator grating correction factor.

Differential scanning calorimetry

Calorimetric measurements were performed as previously reported (17,32) in a Microcal VP differential scanning calorimeter (Microcal, Northampton, MA) at a heating rate of 0.5°C/min. DPPC/POPG/PA MLVs (1 mM) with or without 0.57 mol % KL_4 and in the presence of different amounts of SP-A were loaded in the sample cell of the microcalorimeter with 0.6 ml of buffer A in the reference cell. Experiments were also performed using native surfactant (1 mg/ml, total PL concentration) and LES (1 mg PL/ml) with or without different amounts of SP-A. Three calorimetric scans were collected from each sample between 15°C and 70°C. The standard Microcal Origin software was used for data acquisition and analysis. The excess heat capacity functions were obtained after subtraction of the buffer-buffer baseline.

Giant vesicle preparation

GUVs composed of 1), native surfactant, 2), LES, 3), KL_4-DPPC/POPG/PA, and 4), DPPC/POPC/PA were prepared from lipid samples suspended in buffer B (no organic solvents) as described previously (17,33) by using the electroformation method originally developed by Angelova and Dimitrov (34). GUVs composed of LES, KL_4-DPPC/POPG/PA, and DPPC/POPG/PA were prepared in the absence and presence of different amounts of SP-A. A special temperature-controlled chamber was used for this purpose (35). Briefly ~3 μl of the stock suspension in buffer B in the presence or absence of SP-A were spread in small drops on the surface of each platinum wire. The sample was labeled with Bodipy-PC, the percentage of fluorescent probe in the sample being <0.1 mol % (17). The chamber was then placed under a stream of N_2 and subsequently under low vacuum for 30 min to allow the native material to adsorb onto the platinum wire. An important point in this step is to avoid dehydration of the sample to maintain the integrity of the membranes. Once the material was adsorbed to the platinum wire, aqueous solution was added to the chamber (200 mosM sucrose solution prepared with Millipore-filtered water, 17.5 megohms/cm). The sucrose solution was previously heated to the desired temperature (above the lipid mixture phase transition), and then sufficient volume was added to cover the platinum wires (~300 μl). The platinum wires were connected immediately to a function generator (Digimess FG 100) (Vann Draper Electronics, Derby, UK), and a low frequency alternating current field (sinusoidal wave function with a frequency of 10 Hz and amplitude of 3 V) was applied for 60 min. After vesicle formation, the alternating current field was turned off, and the vesicles were collected with a pipette and transferred into a plastic tube.

Observation of giant vesicles

Aliquots of giant vesicles suspended in sucrose were added to an isoosmolar concentration of glucose solution. The density difference between the interior and exterior of the GUVs induces the vesicles to sink to the bottom of the chamber, and within a few minutes the vesicles are ready to be observed using an inverted microscope. GUV preparations were observed in 12-well plastic chambers (Lab-Tek Brand Products, Naperville, IL). The chamber was located in an inverted confocal microscope (Zeiss LSM 510 META, Zeiss, Jena, Germany) for observation. The excitation wavelength was 488 nm. The temperature was controlled from a water bath connected to a homemade device into which the 12-well plastic chamber was inserted. The temperature was measured inside the sample chamber using a digital thermocouple (model 400B, Omega, Stamford, CT) with a precision of 0.1°C. Unilamellarity was assured by measuring the fluorescence intensity of the equator region as described previously (36).

RESULTS

SP-A concentration-dependent effect on the surface activity of KL_4-surfactant

Fig. 1 shows SP-A effects on the surface activity of DPPC/POPG/PA vesicles with (*right panel*) and without (*left panel*)

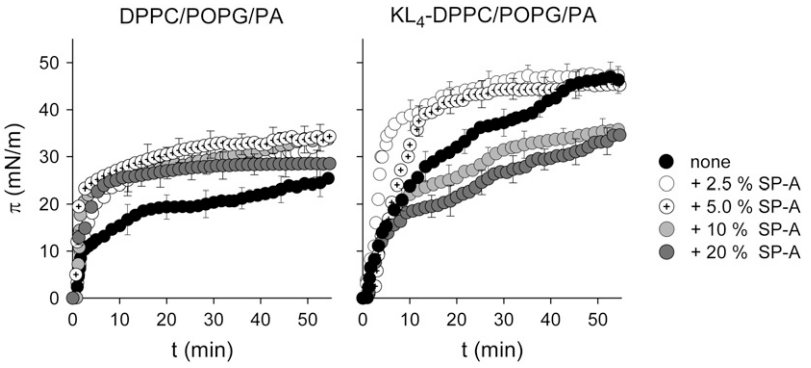

FIGURE 1 Effect of SP-A on the adsorption kinetics of DPPC/POPG/PA membranes with or without 0.57 mol % KL_4 in the presence of 5 mM calcium. PL interfacial adsorption was measured at 25°C after the change in surface pressure as a function of time for samples containing 70 μg PL/ml in the absence (*solid black circle*) and presence of increasing amounts of SP-A: (○) 2.5%, (⊕) 5.0%, (*light shaded circle*) 10%, and (*dark shaded circle*) 20% protein/lipid weight ratio (which correspond to 1.75, 3.5, 7, and 14 μg/ml SP-A, respectively). Values are the mean ± SD of three experiments. Similar results were obtained at 37°C.

0.57 mol % KL_4 in the presence of calcium. The final PL concentration was 70 μg/ml. Addition of different SP-A concentrations to KL_4-containing DPPC/POPG/PA vesicles led to SP-A concentration-dependent changes in the surface properties of these vesicles. Thus at protein/PL weight ratios of 2.5% and 5%, the adsorption rate of these vesicles significantly increased. At SP-A concentrations ≥10 wt %, the surface activity of KL_4-surfactant decreased (Fig. 1, *right panel*). On the contrary, addition of different SP-A concentrations (2.5–20 wt %) to DPPC/POPG/PA membranes without KL_4 significantly enhanced the surface adsorption rate of these vesicles, although the equilibrium pressure was not attained in the absence of KL_4 (Fig. 1, *left panel*).

To find out whether the effects of SP-A on the surface activity of KL_4-surfactant were influenced by the presence of Ca^{2+}, we performed these experiments in the absence of this cation (Fig. 2). We found that the SP-A concentration-dependent effects on the adsorption rate of these vesicles were similar in the absence and presence of calcium. However, in the absence of calcium, it was necessary to increase the amount of surfactant PLs to attain the equilibrium pressure (from 70 μg/ml with Ca^{2+} to 100 μg/ml without Ca^{2+}). Collectively, these data revealed that the SP-A concentration-dependent behavior on the surface activity of KL_4-DPPC/POPG/PA membranes was independent of calcium.

SP-A decreases the ordering effect of KL_4 on surfactant-like membranes

We previously showed that KL_4 has an ordering effect on DPPC/POPG/PA membranes which is dependent on KL_4 concentration (17). Given that the binding of 10 wt % SP-A to KL_4-DPPC/POPG/PA membranes decreased its surface activity, we investigated whether addition of 10 wt % SP-A interfered with the KL_4 ordering effect. Fig. 3 shows that increasing the KL_4 concentration in DPPC/POPG/PA vesicles resulted in significant increase in the steady-state emission anisotropy of DPH incorporated in those vesicles. However, in the presence of 10 wt % SP-A, the KL_4 effect on DPH anisotropy was partially inhibited. Because an increase in the steady-state anisotropy of DPH embedded in vesicles containing KL_4 might be due to greater molecular order of the lipids surrounding DPH and a consequent slowing in DPH rotational diffusion, or to changes in DPH fluorescence lifetime and hence changes in DPH steady-state fluorescence intensity (37), we also measured the fluorescence emission spectra of DPH embedded in KL_4-containing vesicles in the absence and presence of SP-A upon excitation at 360 nm as reported elsewhere (17,32). Within experimental error, no changes were observed in the fluorescence emission of DPH with increasing amounts of peptide, both in the absence and presence of SP-A (data not shown). This allows us to infer

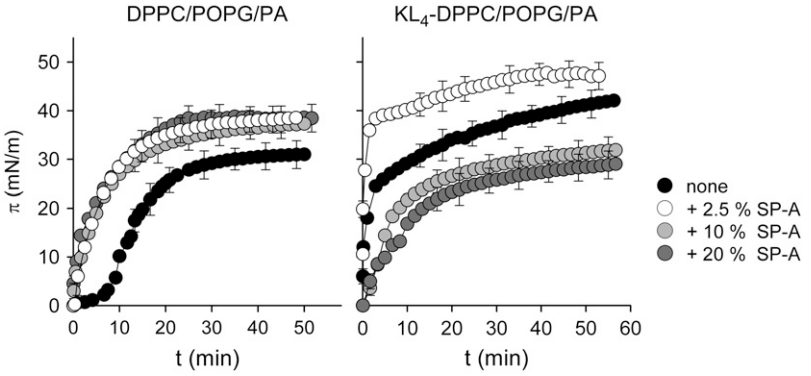

FIGURE 2 Calcium-independent effect of SP-A on the adsorption kinetics of DPPC/POPG/PA membranes with or without 0.57 mol % KL_4. PL interfacial adsorption was measured at 25°C after the change in surface pressure as a function of time for samples containing 100 μg PL/ml in the absence (*solid black circle*) and presence of increasing amounts of SP-A: (○) 2.5%, (*light shaded circle*) 10%, and (*dark shaded circle*) 20% protein/lipid weight ratio (which correspond to 2.5, 5, 10, and 20 μg/ml SP-A, respectively). Values are the mean ± SD of three experiments. Similar results were obtained at 37°C.

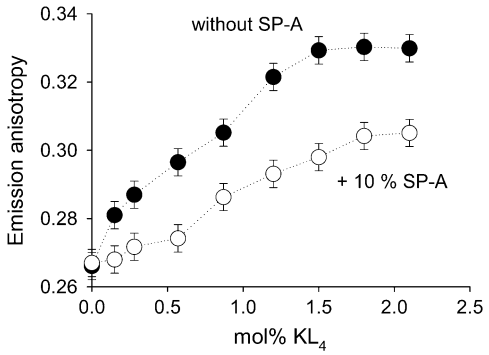

FIGURE 3 Steady-state emission anisotropy of DPH incorporated in DPPC/POPG/PA vesicles (1 mM) containing different concentrations of KL_4 in the absence (●) and presence (○) of SP-A at a protein/lipid weight ratio of 10%. Experiments were performed at 37°C. ($\lambda_x = 360$ nm, $\lambda_m = 430$ nm). Values are the mean ± SD of three experiments.

that the observed increase in DPH anisotropy was due to greater lipid order. Thus results indicate that KL_4 enhanced the lipid order of DPPC/POPG/PA membranes as described previously (17) and SP-A partially inhibited the KL_4-dependent ordering effect.

Next we examined the emission anisotropy of DPH embedded in DPPC/POPG/PA membranes with and without 0.57 mol % KL_4 as a function of SP-A concentration at 37°C. Fig. 4 shows that, in the absence of SP-A, the anisotropy values of DPH were higher in samples containing 0.57 mol % KL_4, which indicates a peptide-promoted increased lipid order (17). Addition of different amounts of SP-A to KL_4-containing DPPC/POPG/PA membranes caused a protein concentration-dependent decrease of the anisotropy values of DPH. At 10 wt % SP-A, the anisotropy values of these membranes with or without KL_4 were similar. The lack of changes in the fluorescence of the probe upon addition of SP-A (data not shown) allows us to infer that SP-A decreases the lipid order of KL_4-surfactant membranes. These results clearly indicate that SP-A interacts with KL_4 incorporated in surfactant-like membranes and that SP-A, at a protein/lipid weight ratio of 10%, abrogates the ordering effect of 0.57 mol % KL_4 on these membranes.

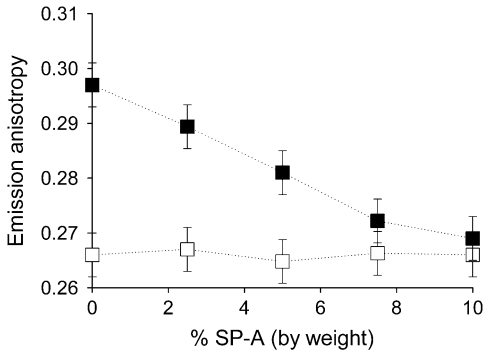

FIGURE 4 Effect of increasing SP-A concentrations on the steady-state emission anisotropy of DPH incorporated in DPPC/POPG/PA membranes (1 mM) in the absence (□) and presence (■) of 0.57 mol % KL_4. Experiments were performed at 37°C ($\lambda_x = 360$ nm, $\lambda_m = 430$ nm). Values are the mean ± SD of three experiments.

SP-A effects on the thermotropic properties of KL_4-surfactant

We used steady-state DPH anisotropy to measure the effect of 10 wt % SP-A on the thermotropic behavior of KL_4-containing membranes (Fig. 5). The fluorescence anisotropy of DPH decreases at temperatures higher than the T_m because the rotational freedom of the probe increases upon PL acyl chain melting (37). The phase transition temperature results from a condition of disorder due to the *trans-gauche*-isomerization of PL acyl chains. In the presence of 10 wt % SP-A, the fluorescence anisotropy of DPH significantly decreased in the solid/fluid transition region of KL_4-DPPC/POPG/PA vesicles, but it slightly increased in the liquid-crystalline phase. This indicates that supraphysiological concentrations of SP-A had a fluidizing effect on KL_4-surfactant at temperatures where the solid/fluid transition occurred. In addition, Fig. 5 shows that SP-A shifted the main transition temperature (T_m) of these vesicles somewhat downward. In the presence of SP-A, the temperature-dependent fluorescence anisotropy change of DPH incorporated into KL_4-DPPC/POPG/PA vesicles resembled that obtained for DPPC/POPG/PA vesicles without KL_4 (data not shown), indicating that SP-A revoked KL_4 effects on DPPC/POPG/PA membranes.

We also used the nonperturbing technique of DSC to probe the effect of SP-A on the thermotropic properties of DPPC/POPG/PA vesicles with and without 0.57 mol % KL_4 (Fig. 6). The thermal transition of KL_4-containing DPPC/POPG/PA membranes was characterized by a complex

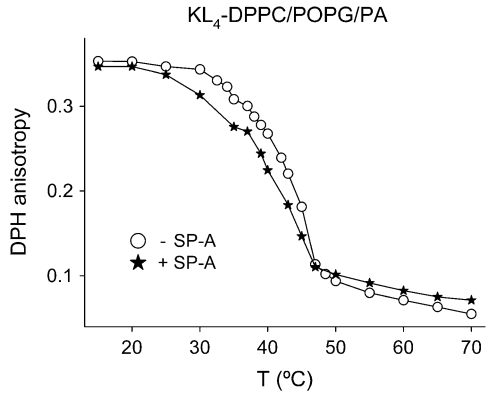

FIGURE 5 Temperature-dependent fluorescence anisotropy of DPH incorporated into KL_4-DPPC/POPG/PA vesicles in the absence (○) and presence (★) of 10 wt % SP-A. The final concentrations of PLs and SP-A were 1 mg/ml and 100 μg/ml, respectively. Values are the mean ± SD of three experiments. The standard deviation for each temperature was too small to be displayed by error bars. ($\lambda_x = 359$ nm, $\lambda_m = 427$ nm).

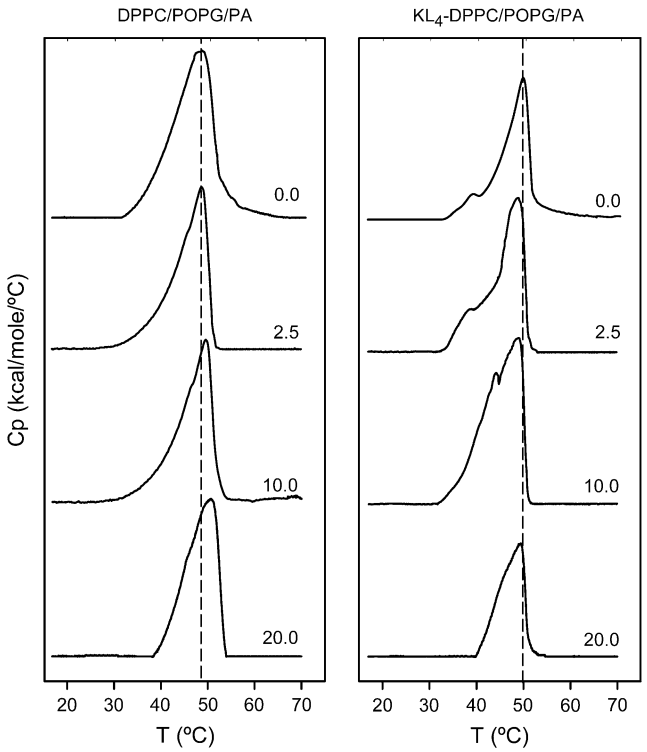

FIGURE 6 Effect of SP-A on DSC heating scans of DPPC/POPG/PA vesicles (1 mM) with or without 0.57 mol % KL_4. The % SP-A/lipid weight ratio is indicated on each thermogram. Calorimetric scans were performed at a rate of 0.5°C/min. For clarity of presentation the tick labels of heat capacity (Cp) are not shown. For each thermogram, the scale of Cp was from 0 to 1.4. One representative experiment of three experiments is shown.

double peak with one relatively sharp peak and a small peak on the low temperature side of the main transition, as described in Saenz et al. (17) (Fig. 6, *right panel*). This double peak is only observed in POPG-containing vesicles (17), indicating that it must be generated by electrostatic interactions between the positively charged lysine residues of KL_4 and the anionic headgroup of POPG (17). Addition of increasing amounts of SP-A perturbed the thermal transition of these KL_4-containing membranes. Thus, whereas the sharp peak shifted slightly to lower temperatures, the height of the small and broad peak increased and shifted to higher temperatures. In the presence of 20 wt % SP-A, the membranes showed an endotherm with a main transition to the liquid-crystalline phase at 49.1 ± 0.1°C. Importantly, whereas KL_4 shifted the main transition temperature (T_m) of surfactant-like vesicles somewhat upward (from 48.5 to 50.0°C for DPPC/POPG/PA) (17), the presence of 10–20 wt % SP-A produced the opposite effect. That is, high concentrations of SP-A shifted the T_m somewhat downward, which is consistent with DPH anisotropy experiments shown in Fig. 5.

Fig. 6 (*left panel*) shows endotherms of DPPC/POPG/PA membranes without KL_4 in the absence and presence of SP-A. In contrast to KL_4-DPPC/POPG/PA vesicles, addition of increasing amounts of SP-A to DPPC/POPG/PA vesicles led to a protein-concentration-dependent increase in the T_m. Moreover, the interaction of SP-A with these vesicles induced some perturbation of the lipid molecular packing so that a narrowing of the phase transition was observed. This could indicate SP-A-mediated stabilization of DPPC-rich assemblies. It is important to note that the thermograms of KL_4-DPPC/POPG/PA vesicles containing SP-A (Fig. 6, *right panel*) were not similar to those of the lipids without KL_4 containing SP-A (Fig. 6, *left panel*). Therefore, it could be inferred that KL_4 is not withdrawn from the membrane by high concentrations of SP-A. Visualization of the effects of SP-A on these vesicles with and without KL_4 (Figs. 7 and 8) confirms this hypothesis.

SP-A effects on the lipid lateral organization of KL_4-containing surfactant-like membranes

Confocal fluorescence microscopy was employed to visualize SP-A effects on the lipid lateral organization of GUVs prepared from DPPC/POPG/PA vesicles with and without 0.57 mol % KL_4. The membranes were doped with the fluorescent probe Bodipy-PC. As described previously (17), DPPC/POPG/PA vesicles, with or without KL_4, showed coexisting bright and dark domains at room temperature, well below their T_m (Figs. 7 and 8). Since Bodipy-PC partitions in the fluid phase (38), dark regions can be ascribed to DPPC-rich solid domains.

Electrostatic repulsions favor the formation of GUVs (39). Given that KL_4 electrostatically interacts with POPG and PA (17,18), decreasing the net negative charge of DPPC/POPG/PA vesicles, the formation of GUVs was reduced in the

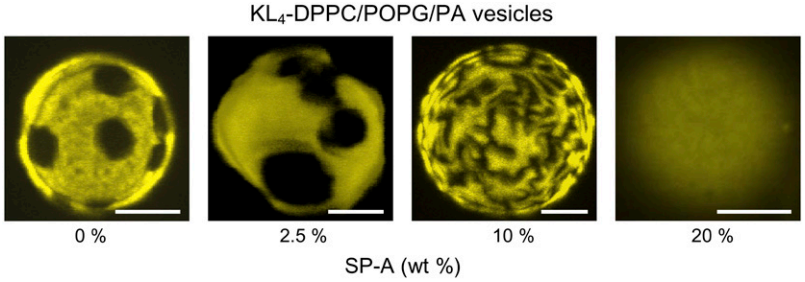

FIGURE 7 SP-A effects on the lipid lateral organization of GUVs prepared from KL_4-DPPC/POPG/PA MLVs doped with the fluorescent probe Bodipy-PC. Images were taken at 25°C. The lipid concentration used was 0.2 mg/ml. SP-A concentrations were 5, 20, and 40 μg/ml. The scale bars correspond to 5 μm.

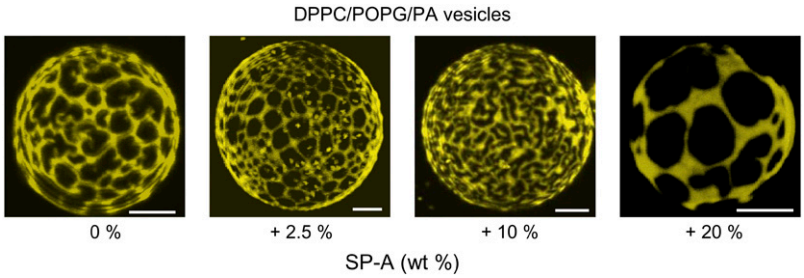

FIGURE 8 SP-A effects on the lipid lateral organization of GUVs prepared from DPPC/POPG/PA MLVs doped with the fluorescent probe Bodipy-PC. Images were taken at 25°C. Lipid and SP-A concentrations were as in Fig. 7. The scale bars correspond to 5 μm.

presence of KL_4, which also induced vesicle aggregation as shown in (17). Therefore, the yield of individual GUVs was very low in the presence of KL_4 (17). Interestingly, the binding of SP-A to KL_4-DPPC/POPG/PA vesicles favored the formation of GUVs. We found that addition of SP-A at concentrations $\geq$2.5 wt % dramatically increased the yield of GUVs and no aggregates were observed. This could be explained by the fact that the binding of SP-A to KL_4-DPPC/POPG/PA vesicles would contribute negative charge to these vesicles. Fig. 7 shows that the shape, size, and number of DPPC-rich solid domains were similar in the absence and presence of 2.5 wt % SP-A. However, increasing the SP-A concentration up to 10 wt % resulted in changes in the shape and size of DPPC-rich solid domains. Solid domains were less apparent and formed narrow stripes as shown in Fig. 7. At a protein/lipid weight ratio of 20 wt %, the fluorescence of the dye was uniform over the GUV surface, indicating either that there is a single phase or that solid domains have much smaller dimensions than the optical resolution (Fig. 7). Collectively, these results indicated that supraphysiological concentrations of SP-A increased the ratio of fluid/solid domains of KL_4-DPPC/POPG/PA membranes, resulting in disappearance of the solid/fluid phase coexistence distinctive of these vesicles.

On the other hand, we did not find this SP-A fluidizing effect on GUVs prepared from DPPC/POPG/PA in the absence of KL_4 (Fig. 8). We previously reported that SP-A recognizes the lipid in the gel phase (40) but can only penetrate into the membrane interface in lipid-packing defects at solid/fluid boundaries (41–43). Here we found that addition of 2.5 wt % SP-A to DPPC/POPG/PA vesicles increased the number of solid domains and decreased their size (Fig. 8). These results are consistent with our previous findings on SP-A's ability to reduce the size and increase the number of condensed domains of DPPC monolayers (41). Increasing the SP-A concentration up to 10 wt % led to changes in the shape of the solid domains, which became elongated as connected bands on the surface of these vesicles. The change in the size and shape of the DPPC-rich solid domain of DPPC/POPG/PA vesicles induced by 2.5–10 wt % SP-A can be explained by electrostatic repulsion between SP-A and both POPG and PA, which would favor strip-like structures. This hypothesis was confirmed by envisioning the effect of 10 wt % SP-A on DPPC/POPC/PA vesicles or DPPC/POPG/PA vesicles in the presence of calcium (data not shown). In both cases, the sizes of the DPPC-rich domains were not reduced and the shapes were circular. Separation of the solid phase into more numerous domains in vesicles containing anionic PLs increases the distance between their dipole moments, resulting in lower electrostatic repulsion (44,45). Interestingly, further increasing the SP-A concentration up to 20 wt % resulted in the formation of larger DPPC-rich solid domains (Fig. 8). The formation of such large circular solid domains at high SP-A concentrations (20% by weight) is intriguing. It is possible that, at 20 wt % SP-A, the electrostatic repulsions between the protein and negatively charged lipids (POPG and PA) partially prevent the interaction of the protein with the membrane. On the contrary, in KL_4-DPPC/POPG/PA vesicles, the charge neutralization of the membrane induced by KL_4 would favor the interaction of SP-A with the vesicles, leading to membrane perturbation and disappearance of the solid/fluid phase coexistence.

SP-A effects on the surface activity and physical properties of porcine lipid extract surfactant

We also studied the effect of SP-A on the surface activity and physical properties of a LES obtained from porcine bronchoalveolar fluid to compare SP-A effects on KL_4-surfactant with those on natural surfactant. Porcine LES consisted of PL vesicles that contain ~10–15 mol % Ch and the hydrophobic surfactant proteins SP-B and SP-C as the only protein constituents. The PL composition of these vesicles was PC (79%), phosphatidylinositol (8%), PG (4%), phosphatidylserine (2%), phosphatidylethanolamine (3%), sphingomyelin (2.5%), and lysophosphatidylcholine (1.5%) as previously reported (46). The content of hydrophobic proteins in porcine LES, detected by ELISA, was 1.7 nmol SP-B/μmol PL and 3.5 nmol SP-C/μmol PL. We did not measure the content of DPPC, the molecular species of PC distinctive of lung surfactant, but it is reported that DPPC constitutes 60 mol % of the total lung surfactant PC from adult pigs (47).

Fig. 9 *A* shows surface adsorption kinetics of porcine LES with and without SP-A at a concentration of surfactant PL of 50 μg/ml. This low concentration of surfactant material was deliberately chosen to obtain a measurable effect of SP-A on

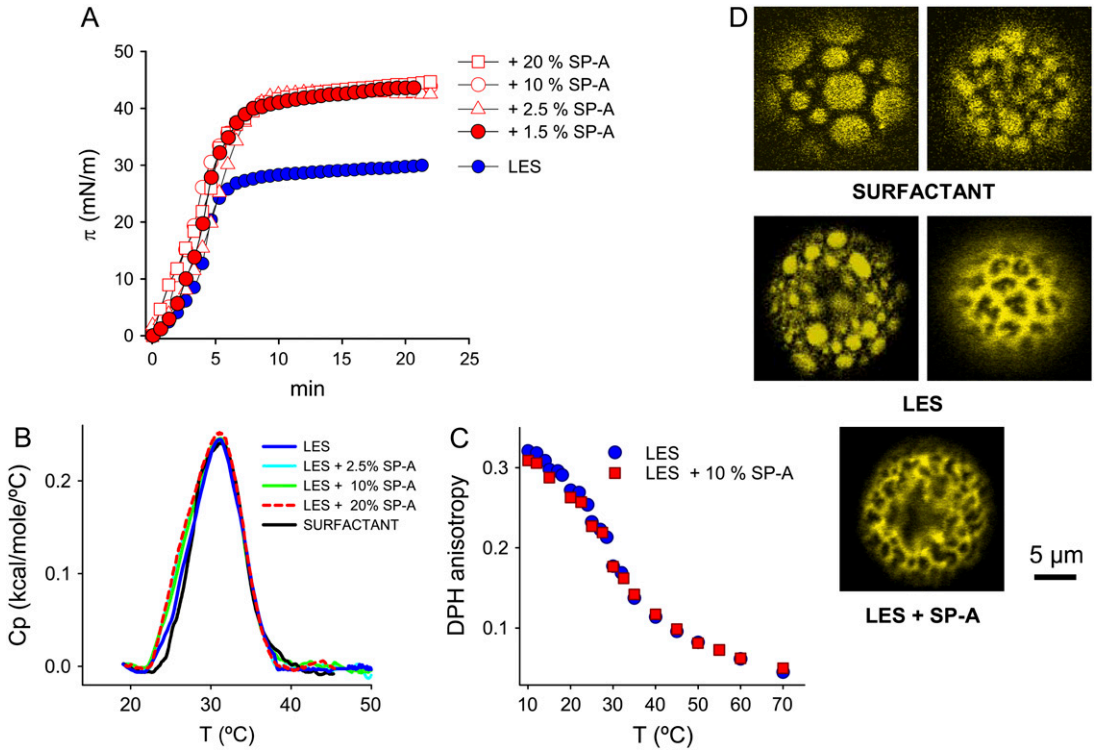

FIGURE 9 Effect of SP-A on porcine LES, which contains the entire lipid associated with surfactant plus the hydrophobic surfactant proteins SP-B and SP-C. (*A*) Adsorption kinetics of LES (50 μg/ml) in the absence and presence of 1.5, 2.5, 10, and 20 wt % SP-A; (*B*) DSC heating scans of porcine lung surfactant (1 mg PL/ml) and porcine LES (1 mg PL/ml) in the absence and presence of 2.5, 10, and 20 wt % SP-A; (*C*) Effect of 10 wt % SP-A on the temperature-dependent fluorescence anisotropy of DPH incorporated into LES (1 mg/ml). Similar results were found with 20 wt % SP-A. In panels *A–C* one representative experiment of two experiments is shown; (*D*) Representative confocal fluorescence images of GUVs composed of porcine lung surfactant, LES, and LES plus SP-A. The giant vesicles were labeled with the fluorescent probe Bodipy-PC, which partitions in the fluid phase. Images were taken at 25°C. The lipid concentration used was 0.2 mg/ml. The SP-A concentration was 20 μg/ml. The scale bar corresponds to 5 μm.

the rate of change of surface pressure. Fig. 9 *A* shows that SP-A concentrations as low as 1.5 wt % greatly enhanced the surface adsorption rate, reaching the equilibrium surface pressure (40–45 mN/m) at 8 min. Greater SP-A concentrations (2.5, 10, or 20 wt %) had no further effect on surface adsorption. In contrast to the results found with KL_4-surfactant, supraphysiological concentrations of SP-A (≥10 wt %) had no inhibitory effect on the surface adsorption rate of LES. These results are consistent with previous data that demonstrated an SP-A-promoted increase of the surface activity of lipid extracts from animal-derived surfactants (9,26,27,48). This effect seemed to be independent of the amount of SP-A added.

Fig. 9 *B* shows a representative DSC experiment obtained from porcine lung surfactant, LES, and LES plus different SP-A concentrations (2.5–20 wt %). The thermograms were almost similar. They were characterized by a broad melting event with low melting enthalpy likely due to the lipid composition of lung surfactant membranes, which contained Ch. Results in Fig. 9 *B* indicate that neither the absence of SP-A in LES nor the addition of 10 or 20 wt % SP-A to LES influenced the thermotropic behavior of these membranes. Similar results were found using steady-state DPH anisotropy to measure the effect of 10 or 20 wt % SP-A on the thermotropic behavior of LES (Fig. 9 *C*).

Fig. 9 *D* shows images of single GUVs composed of porcine lung surfactant, porcine LES, and LES plus 10 wt % SP-A doped with the fluorescent probe Bodipy-PC. GUVs from lung surfactant and LES exhibited the typical liquid ordered/liquid disordered-like phase coexistence previously reported (33), which is characterized by the presence of fluorescent round domains over a dark background (33). Alternatively, some images of surfactant and LES showed dark solid domains over a fluorescent background. Images from surfactant and LES were very similar. This suggests that the absence of the water-soluble fraction of surfactant in LES (which included SP-A) had no effect on the lipid lateral organization of these membranes, as reported previously (33). Phase coexistence is a characteristic of such membranes and occurs at room and physiological temperatures (33,49). In contrast to the results found with KL_4-surfactant, supraphysiological concentrations of SP-A did not increase the ratio of fluid/solid domains in LES and did not cause disappearance of the solid/fluid phase coexistence typical of these vesicles. Collectively, these results indicate that, in opposition to the results found with KL_4-surfactant, supraphysiological

concentrations of SP-A did not have a fluidizing effect of LES and improved the surface adsorption of these surfactant membranes.

DISCUSSION

It is known that maintenance of a stable DPPC-rich surface film is essential for respiration and requires the surface adsorption process. Surface adsorption involves rapid insertion of PLs into the expanding film, which requires conversion from bilayer aggregates to interfacial film (50,51). Both SP-B and SP-C promote adsorption of PLs from vesicles in the aqueous subphase into the air-liquid interface, but the mechanism by which surfactant hydrophobic proteins promotes bilayer-monolayer transitions is not understood (50, 51). On the other hand, studies with lipid extracts from lung surfactant indicate that at low surfactant concentrations small amounts of SP-A enhance the rate of surface adsorption (48). Low or high (2–20 wt %) SP-A concentrations enhance adsorption of lipids along the air-water interface in the presence of the hydrophobic surfactant protein SP-B (8,52,53). In this study we have examined the effect of different concentrations of SP-A on the surface adsorption of a synthetic lung surfactant based upon the cationic and hydrophobic 21-residue peptide KL_4 inserted in DPPC/POPG/PA vesicles as well as on the physical properties and lateral lipid organization of these membranes.

KL_4 mimics the positive charge and hydrophobic residue distribution of SP-B (16). KL_4, as well as SP-B and SP-C, promotes rapid adsorption of surfactant-like vesicles to an air-water interface (16–19). This cationic peptide interacts with anionic PLs (16–18) and has an ordering effect on surfactant-like vesicles (17) comparable to that of SP-B (54,55). KL_4 causes immiscibility between DPPC and POPG in bilayers (17) and monolayers (18), promoting lateral phase separation, which could explain the better overall surface adsorption of KL_4-containing membranes. In this study we found that concentrations of SP-A $\geq$10 wt % 1), decreased the rate of surface adsorption of KL_4-DPPC/POPG/PA membranes; 2), abrogated KL_4-induced ordering effect in these membranes; and 3), increased miscibility between DPPC and POPG, decreasing DPPC-rich solid domain fraction. This caused disappearance of solid/fluid phase coexistence of KL_4-DPPC/POPG/PA vesicles at high SP-A concentrations.

Our results suggest specific interactions between SP-A and KL_4 and also between SP-A and surfactant-like membranes, which finally influence the surface behavior of the whole system. Charge neutralization of the membrane induced by KL_4 would favor the interaction of negatively charged SP-A with vesicles, leading to membrane perturbation and disappearance of the solid/fluid phase coexistence. The strong effects of supraphysiological concentrations of SP-A on the physical properties and lateral lipid organization of KL_4-DPPC/POPG/PA membranes might explain why SP-A at high concentrations decreased the surface activity of KL_4-surfactant. We previously reported that the physical state of the membrane plays a critical role in the surface adsorption process mediated by low KL_4 concentrations (0.57 mol %) (17). Thus KL_4-DPPC/POPG/PA and KL_4-DPPC/POPC/PA vesicles, which show well-defined solid/fluid phase coexistence at temperatures below their T_m, exhibit very rapid surface adsorption. In contrast, more fluid (DPPC/POPG) or excessively rigid (DPPC/PA) KL_4-containing membranes are unable to adsorb rapidly onto an air-water interface (17). The fact that SP-A, at high concentrations, led to a visible disappearance of the solid/fluid phase coexistence distinctive of KL_4-DPPC/POPG/PA vesicles and inhibited the surface activity of these vesicles argues that phase coexistence has an important role in promoting surface adsorption. This is consistent with the fact that phase coexistence of liquid-ordered and liquid-disordered phases exists in GUVs prepared from porcine lung surfactant or LES as previously reported (33). In contrast to the results found with KL_4-surfactant, addition of high SP-A concentrations to LES did not affect the phase coexistence characteristic of LES and significantly enhanced the surface adsorption rate of these membranes. Together, these results suggest that phase coexistence in surfactant membranes is important for the surface adsorption process. It is possible that the presence of fluid phases in the lateral organization of DPPC-rich surfactant bilayers facilitates fusion of vesicles to the air-water interface, which implies bilayer disruption. Unsaturated PLs, present in fluid domains, might form transient, negatively curved structures in the bilayer-monolayer transition as proposed by Hall and co-workers (51,56,57) or rapidly flip to the air-water interface. This process could be mediated by surfactant hydrophobic peptides (SP-B and/or SP-C) or KL_4, all of which are located in the fluid phase (17,18,33).

On the other hand, physiological concentrations of SP-A did not perturb phase coexistence of KL_4-DPPC/POPG/PA membranes and significantly improved the rate of surface adsorption of KL_4-surfactant. Importantly, SP-A at either low or high concentrations (2.5–20 wt %) enhanced the surface activity of DPPC/POPG/PA membranes without KL_4, although the equilibrium pressure was not reached in the absence of the peptide. Thus SP-A concentration-dependent behavior on surface adsorption of KL_4-surfactant seems to depend on electrostatic interactions between SP-A and KL_4, which block KL_4 effects on DPPC/POPG/PA membranes. The mechanism by which SP-A at physiological concentrations enhances the surface adsorption rate of KL_4-DPPC/POPG/PA membranes as well as of lipid extracts from animal-derived surfactants (9,26,27,48) or lipid mixtures containing SP-B or SP-B plus SP-C (8,52,53) is unknown, but it must be related to the ability of SP-A to bind DPPC over other lipids (40). SP-A could promote stabilization of DPPC-rich assemblies. It is known that SP-A stimulates DPPC-specific surface adsorption from DPPC/POPG/POPC vesicles containing SP-B (53). It is possible that the role of SP-A is just to increase immiscibility between DPPC and POPG or POPC,

whereas the role of SP-B or KL_4, which specifically interact with PG (16–18,54,58), is not only to cause immiscibility between DPPC and POPG but also to promote the bilayer-monolayer transition and hence overall spreadability of the mixture.

In the future, synthetic surfactants containing recombinant human SP-A might be routinely used in the treatment of lung diseases, given that replacement surfactants containing 5 wt % SP-A are superior to those surfactants lacking this component (13,14) and that SP-A helps to prevent surfactant inhibition by serum proteins (9). The finding that enrichment of KL_4-surfactant with SP-A at concentrations above a certain limiting value may not improve but reduce the activity of this synthetic surfactant is relevant to the preparation of SP-A-enriched synthetic surfactants based upon KL_4 or other cationic and hydrophobic synthetic peptides that mimic surface properties of SP-B.

This work was supported by grants from Fondo de Investigación Sanitaria (03/0137), FMM-2005, SAF2006-04434, and by Laboratorios Dr. Esteve, S. A. (Barcelona, Spain). Research in the laboratory of L.A.B. is funded by a grant from Statens Naturvidenskabellge Forskningsrad, Denmark (21-03-0569).

REFERENCES

1. Johansson, J., and T. Curstedt. 1997. Molecular structures and interactions of pulmonary surfactant components. *Eur. J. Biochem.* 244:675–693.
2. Hawgood, S. 1997. Surfactant: composition, structure, and metabolism. *In* The Lung: Scientific Foundations, 2nd Ed. R. G. Crystal, J. B. West, P. J. Barnes, and E. R. Weibel, editors. Lippincott-Raven Publishers, Philadelphia, PA. 557–571.
3. Wright, J. R. 2005. Immunoregulatory functions of surfactant proteins. *Nature Rev. Immunol.* 5:58–68.
4. Whitsett, J. A., and T. E. Weaver. 2002. Hydrophobic surfactant proteins in lung function and disease. *N. Engl. J. Med.* 347:2141–2148.
5. Robertson, B., and H. L. Halliday. 1998. Principles of surfactant replacement. *Biochim. Biophys. Acta.* 1408:346–361.
6. Casals, C., and I. García-Verdugo. 2005. Molecular and functional properties of surfactant protein A. *In* Developments in Lung Surfactant Dysfunction in Lung Biology in Health and Disease. K. Nag, editor. Marcel Dekker, New York. 55–84.
7. Casals, C. 2001. Role of surfactant protein A (SP-A)/lipid interactions for SP-A functions in the lung. *Pediatr. Pathol. Mol. Med.* 20:249–268.
8. Hawgood, S., B. J. Benson, J. Schilling, D. Damm, J. A. Clements, and R. T. White. 1987. Nucleotide and amino acid sequences of pulmonary surfactant protein SP 18 and evidence for cooperation between SP 18 and SP 28-36 in surfactant lipid adsorption. *Proc. Natl. Acad. Sci. USA.* 84:66–70.
9. Cockshutt, A. M., J. Weitz, and F. Possmayer. 1990. Pulmonary surfactant-associated protein A enhances the surface activity of lipid extract surfactant and reverses inhibition by blood proteins in vitro. *Biochemistry.* 29:8424–8429.
10. Ikegami, M., T. R. Korfhagen, J. A. Whitsett, M. D. Bruno, S. E. Wert, K. Wada, and A. H. Jobe. 1998. Characteristics of surfactant from SP-A-deficient mice. *Am. J. Physiol.* 275:L247–L254.
11. Ingenito, E. P., L. Mark, J. Morris, F. F. Espinosa, R. D. Kamm, and M. Johnson. 1999. Biophysical characterization and modeling of lung surfactant components. *J. Appl. Physiol.* 86:1702–1714.
12. Bernhard, W., J. Mottaghian, A. Gebert, G. A. Rau, H. von Der Hardt, and C. F. Poets. 2000. Commercial versus native surfactants. Surface activity, molecular components, and the effect of calcium. *Am. J. Respir. Crit. Care Med.* 162:1524–1533.
13. Yamada, T., M. Ikegami, B. L. Tabor, and A. H. Jobe. 1990. Effects of surfactant protein-A on surfactant function in preterm ventilated rabbits. *Am. Rev. Respir. Dis.* 142:754–757.
14. Sun, B., T. Curstedt, G. Lindgren, B. Franzen, A. A. Alaiya, A. Calkovska, and B. Robertson. 1997. Biophysical and physiological properties of a modified porcine surfactant enriched with surfactant protein A. *Eur. Respir. J.* 10:1967–1974.
15. Robertson, B., J. Johansson, and T. Curstedt. 2000. Synthetic surfactants to treat neonatal lung disease. *Mol. Med. Today.* 6:119–124.
16. Cochrane, C. G., and S. D. Revak. 1991. Pulmonary surfactant protein B (SP-B): structure-function relationships. *Science.* 254:566–568.
17. Saenz, A., O. Canadas, L. A. Bagatolli, M. E. Johnson, and C. Casals. 2006. Physical properties and surface activity of surfactant-like membranes containing the cationic and the hydrophobic peptide KL4. *FEBS J.* 273:2515–2527.
18. Ma, J., S. Koppenol, H. Yu, and G. Zografi. 1998. Effects of a cationic and hydrophobic peptide, KL4, on model lung surfactant lipid monolayers. *Biophys. J.* 74:1899–1907.
19. Nilsson, G., M. Gustafsson, G. Vandenbussche, E. Veldhuizen, W. J. Griffiths, J. Sjovall, H. P. Haagsman, J. M. Ruysschaert, B. Robertson, T. Curstedt, and J. Johansson. 1998. Synthetic peptide-containing surfactants—evaluation of transmembrane versus amphipathic helices and surfactant protein C poly-valyl to poly-leucyl substitution. *Eur. J. Biochem.* 255:116–124.
20. Cochrane, C. G., S. D. Revak, T. A. Merritt, G. P. Heldt, M. Hallman, M. D. Cunningham, D. Easa, A. Pramanik, D. K. Edwards, and M. S. Alberts. 1996. The efficacy and safety of KL4-surfactant in preterm infants with respiratory distress syndrome. *Am. J. Respir. Crit. Care Med.* 153:404–410.
21. Sinha, S. K., T. Lacaze-Masmonteil, A. Valls i Soler, T. E. Wiswell, J. Gadzinowski, J. Hajdu, G. Bernstein, M. Sanchez-Luna, R. Segal, C. J. Schaber, J. Massaro, and R. d'Agostino. 2005. A multicenter, randomized, controlled trial of lucinactant versus poractant alfa among very premature infants at high risk for respiratory distress syndrome. *Pediatrics.* 115:1030–1038.
22. Cochrane, C. G., S. D. Revak, T. A. Merritt, I. U. Schraufstatter, R. C. Hoch, C. Henderson, S. Andersson, H. Takamori, and Z. G. Oades. 1998. Bronchoalveolar lavage with KL4-surfactant in models of meconium aspiration syndrome. *Pediatr. Res.* 44:705–715.
23. Wiswell, T. E., G. R. Knight, N. N. Finer, S. M. Donn, H. Desai, W. F. Walsh, K. C. Sekar, G. Bernstein, M. Keszler, V. E. Visser, T. A. Merritt, F. L. Mannino, L. Mastrioianni, B. Marcy, S. D. Revak, H. Tsai, and C. G. Cochrane. 2002. A multicenter, randomized, controlled trial comparing Surfaxin (Lucinactant) lavage with standard care for treatment of meconium aspiration syndrome. *Pediatrics.* 109:1081–1087.
24. Wiswell, T. E., R. M. Smith, L. B. Katz, L. Mastroianni, D. Y. Wong, D. Willms, S. Heard, M. Wilson, R. D. Hite, A. Anzueto, S. D. Revak, and C. G. Cochrane. 1999. Bronchopulmonary segmental lavage with Surfaxin (KL(4)-surfactant) for acute respiratory distress syndrome. *Am. J. Respir. Crit. Care Med.* 160:1188–1195.
25. Ruano, M. L. F., J. Perez-Gil, and C. Casals. 1998. Effect of acidic pH on the structure and lipid binding properties of porcine surfactant protein A. Potential role of acidification along its exocytic pathway. *J. Biol. Chem.* 273:15183–15191.
26. Sanchez-Barbero, F., J. Strassner, R. Garcia-Canero, W. Steinhilber, and C. Casals. 2005. Role of the degree of oligomerization in the structure and function of human surfactant protein A. *J. Biol. Chem.* 280:7659–7670.
27. Garcia-Verdugo, I., F. Sanchez-Barbero, F. U. Bosch, W. Steinhilber, and C. Casals. 2003. Effect of hydroxylation and N187-linked glycosylation on molecular and functional properties of recombinant human surfactant protein A. *Biochemistry.* 42:9532–9542.
28. Casals, C., L. Herrera, E. Miguel, P. Garcia-Barreno, and A. M. Municio. 1989. Comparison between intra- and extracellular surfactant

in respiratory distress induced by oleic acid. *Biochim. Biophys. Acta.* 1003:201–203.

29. Casals, C., A. Varela, M. L. Ruano, F. Valino, J. Perez-Gil, N. Torre, E. Jorge, F. Tendillo, and J. L. Castillo-Olivares. 1998. Increase of C-reactive protein and decrease of surfactant protein A in surfactant after lung transplantation. *Am. J. Respir. Crit. Care Med.* 157:43–49.
30. Oviedo, J. M., F. Valiño, I. Plasencia, A. G. Serrano, C. Casals, and J. Pérez-Gil. 2001. Quantitation of pulmonary surfactant protein SP-B in the absence or presence of phospholipids by enzyme-linked immunosorbent assay. *Anal. Biochem.* 293:78–87.
31. Schmidt, R., W. Steinhilber, C. Ruppert, C. Daum, F. Grimminger, W. Seeger, and A. Gunther. 2002. An ELISA technique for quantification of surfactant apoprotein (SP)-C in bronchoalveolar lavage fluid. *Am. J. Respir. Crit. Care Med.* 165:470–474.
32. Canadas, O., R. Guerrero, R. Garcia-Canero, G. Orellana, M. Menendez, and C. Casals. 2004. Characterization of liposomal tacrolimus in lung surfactant-like phospholipids and evaluation of its immunosuppressive activity. *Biochemistry.* 43:9926–9938.
33. Bernardino de la Serna, J., J. Perez-Gil, A. C. Simonsen, and L. A. Bagatolli. 2004. Cholesterol rules: direct observation of the coexistence of two fluid phases in native pulmonary surfactant membranes at physiological temperatures. *J. Biol. Chem.* 279:40715–40722.
34. Angelova, M. I., and D. S. Dimitrov. 1986. Liposome electroformation. *Faraday Discuss. Chem. Soc.* 81:303–311.
35. Bagatolli, L. A., and E. Gratton. 2000. Two photon fluorescence microscopy of coexisting lipid domains in giant unilamellar vesicles of binary phospholipid mixtures. *Biophys. J.* 78:290–305.
36. Akashi, K., H. Miyata, H. Itoh, and K. Kinosita Jr. 1996. Preparation of giant liposomes in physiological conditions and their characterization under an optical microscope. *Biophys. J.* 71:3242–3250.
37. Lakowicz, J. R. 1999. Principles of Fluorescence Spectroscopy. Kluwer Academic/Plenum, New York.
38. Huang, N. N., K. Florine-Casteel, G. W. Feigenson, and C. Spink. 1988. Effect of fluorophore linkage position of *n*-(9-anthroyloxy) fatty acids on probe distribution between coexisting gel and fluid phospholipid phases. *Biochim. Biophys. Acta.* 939:124–130.
39. Akashi, K., H. Miyata, H. Itoh, and K. Kinosita Jr. 1998. Formation of giant liposomes promoted by divalent cations: critical role of electrostatic repulsion. *Biophys. J.* 74:2973–2982.
40. Casals, C., E. Miguel, and J. Perez-Gil. 1993. Tryptophan fluorescence study on the interaction of pulmonary surfactant protein A with phospholipid vesicles. *Biochem. J.* 296:585–593.
41. Ruano, M. L. F., K. Nag, L. A. Worthman, C. Casals, J. Perez-Gil, and K. M. Keough. 1998. Differential partitioning of pulmonary surfactant protein SP-A into regions of monolayers of dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylcholine/dipalmitoylphosphatidylglycerol. *Biophys. J.* 74:1101–1109.
42. Ruano, M. L. F., K. Nag, C. Casals, J. Perez-Gil, and K. M. Keough. 1999. Interactions of pulmonary surfactant protein A with phospholipid monolayers change with pH. *Biophys. J.* 77:1469–1476.
43. Worthman, L. A., K. Nag, N. Rich, M. L. F. Ruano, C. Casals, J. Perez-Gil, and K. M. Keough. 2000. Pulmonary surfactant protein A interacts with gel-like regions in monolayers of pulmonary surfactant lipid extract. *Biophys. J.* 79:2657–2666.
44. McConnell, H. M. 1991. Structures and transitions in lipid monolayers at the air-water interface. *Annu. Rev. Phys. Chem.* 42:171–195.
45. McConnell, H. M., and V. T. Moy. 1988. Shapes of finite two-dimensional lipid domains. *J. Phys. Chem.* 98:4520–4525.
46. Casals, C., M. L. F. Ruano, E. Miguel, P. Sanchez, and J. Perez-Gil. 1994. Surfactant protein-C enhances lipid aggregation activity of surfactant protein-A. *Biochem. Soc. Trans.* 22:370S.
47. Bernhard, W., S. Hoffmann, H. Dombrowsky, G. A. Rau, A. Kamlage, M. Kappler, J. Haitsma, J. Freihorst, H. von der Hardt, and C. F. Poets. 2001. Phosphatidylcholine molecular species in lung surfactant: composition in relation to respiratory rate and lung development. *Am. J. Respir. Cell Mol. Biol.* 25:725–731.
48. Schurch, S., F. Possmayer, S. Cheng, and A. M. Cockshutt. 1992. Pulmonary SP-A enhances adsorption and appears to induce surface sorting of lipid extract surfactant. *Am. J. Physiol.* 263:L210–L218.
49. Nag, K., J. S. Pao, R. R. Harbottle, F. Possmayer, N. O. Petersen, and L. A. Bagatolli. 2002. Segregation of saturated chain lipids in pulmonary surfactant films and bilayers. *Biophys. J.* 82:2041–2051.
50. Walters, R. W., R. R. Jenq, and S. B. Hall. 2000. Distinct steps in the adsorption of pulmonary surfactant to an air-liquid interface. *Biophys. J.* 78:257–266.
51. Piknova, B., V. Schram, and S. B. Hall. 2002. Pulmonary surfactant: phase behavior and function. *Curr. Opin. Struct. Biol.* 12:487–494.
52. Possmayer, F., K. Nag, K. Rodriguez, R. Qanbar, and S. Schürch. 2001. Surface activity in vitro: role of surfactant proteins. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 129:209–220.
53. Veldhuizen, E. J., J. J. Batenburg, L. M. van Golde, and H. P. Haagsman. 2000. The role of surfactant proteins in DPPC enrichment of surface films. *Biophys. J.* 79:3164–3171.
54. Baatz, J. E., V. Sarin, D. R. Absolom, C. Baxter, and J. A. Whitsett. 1991. Effects of surfactant-associated protein SP-B synthetic analogs on the structure and surface activity of model membrane bilayers. *Chem. Phys. Lipids.* 60:163–178.
55. Vincent, J. S., S. D. Revak, C. G. Cochrane, and I. W. Levin. 1991. Raman spectroscopic studies of model human pulmonary surfactant systems: phospholipid interactions with peptide paradigms for the surfactant protein SP-B. *Biochemistry.* 30:8395–8401.
56. Schram, V., and S. B. Hall. 2004. SP-B and SP-C alter diffusion in bilayers of pulmonary surfactant. *Biophys. J.* 86:3734–3743.
57. Biswas, S. C., S. B. Rananavare, and S. B. Hall. 2005. Effects of gramicidin-A on the adsorption of phospholipids to the air-water interface. *Biochim. Biophys. Acta.* 1717:41–49.
58. Perez-Gil, J., C. Casals, and D. Marsh. 1995. Interactions of hydrophobic lung surfactant proteins SP-B and SP-C with dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylglycerol bilayers studied by electron spin resonance spectroscopy. *Biochemistry.* 34:3964–3971.