Interaction of Pulmonary Surfactant Protein SP-A with DPPC/Egg-PG Bilayers

Michael R. Morrow,* Nidal Abu-Libdeh,* June Stewart,† and Kevin M. W. Keough†‡
*Department of Physics and Physical Oceanography, †Department of Biochemistry, and
‡Discipline of Pediatrics, Memorial University of Newfoundland, St. John's, Newfoundland, A1B 3X9 Canada

ABSTRACT In the mixture of lipids and proteins which comprise pulmonary surfactant, the dominant protein by mass is surfactant protein A (SP-A), a hydrophilic glycoprotein. SP-A forms octadecamers that interact with phospholipid bilayer surfaces in the presence of calcium. Deuterium NMR was used to characterize the perturbation by SP-A, in the presence of 5 mM Ca^{2+}, of dipalmitoyl phosphatidylcholine (DPPC) properties in DPPC/egg-PG (7:3) bilayers. Effects of SP-A were uniformly distributed over the observed DPPC population. SP-A reduced DPPC chain orientational order significantly in the gel phase but only slightly in the liquid-crystalline phase. Quadrupole echo decay times for DPPC chain deuterons were sensitive to SP-A in the liquid-crystalline mixture but not in the gel phase. SP-A reduced quadrupole splittings of DPPC choline β-deuterons but had little effect on choline α-deuteron splittings. The observed effects of SP-A on DPPC/egg-PG bilayer properties differ from those of the hydrophobic surfactant proteins SP-B and SP-C. This is consistent with the expectation that SP-A interacts primarily at bilayer surfaces.

INTRODUCTION

Reduction of surface tension at the alveolar air-water interface (Von Neergaard, 1929) results from the presence of a protein-lipid surfactant complex that is derived from material secreted into the calcium-containing alveolar hypophase as lamellar bodies by type II cells (Goerke, 1998). Proteins account for 5–10% of the total surfactant weight (Pérez-Gil and Keough, 1998; Goerke, 1998). Much of the remainder is phospholipids including DPPC (~40%) and phosphatidylglycerol (PG) (up to 10%) (Yu et al., 1983; Goerke, 1998; Veldhuizen et al., 1998). Surfactant protein A (SP-A) is a hydrophilic glycoprotein with a collagen-like N-terminal domain and variable glycosylation of the C-terminal region that may contribute to the surface activity of pulmonary surfactant or may play a role in host defense (King and Clements, 1972; Hawgood et al., 1985; Drickamer et al., 1986; McCormack, 1998). Trimers of SP-A associate into an octadecamer with a molecular mass of ~700 kDa (Voss et al., 1988). In the presence of Ca^{2+}, SP-A interacts with phosphatidylcholine bilayers through its C-terminal region (Palaniyar et al., 1998). Williams et al. (1991) examined bilayer organization for DPPC/egg PG (7:3) mixtures containing various combinations of surfactant proteins. The presence of SP-A and calcium resulted in multilamellar structures in which membranes were uniformly separated by ~20 nm. The observation of particles between layers and the similarity of the 20-nm interbilayer spacing with the size of the SP-A octadecamer led to the suggestion that SP-A between bilayers was responsible for maintaining this parallel alignment of layers.

Natural surfactant also contains two hydrophobic proteins. Pulmonary surfactant protein B (SP-B) is a 79-residue hydrophobic protein that forms disulfide-linked homodimers with regions of amphipatic α-helix (Curstedt et al., 1990; Hawgood et al., 1998). Pulmonary surfactant protein C (SP-C) is a 35-residue protein with a 23-residue α-helix in the C-terminal part of the protein (Curstedt et al., 1990; Johansson 1998). SP-C is likely capable of spanning bilayers (Morrow et al. 1993).

Natural surfactant has been observed to form a structure, tubular myelin, that appears as a square lattice in electron micrographs (Benson et al., 1984; Putman et al., 1996). This material may be a precursor of surface active material or may act as a reservoir for that material (Gil and Reiss, 1973; Sanders et al., 1980; Sen et al., 1988). Formation of tubular myelin in vitro appears to require DPPC, unsaturated PG, SP-A, SP-B, and calcium (Suzuki et al., 1989; Williams et al., 1991; Poulain et al., 1992). The edges of the lattice squares in synthetic tubular myelin have been reported to be ~50–55 nm although larger squares were observed at higher SP-A concentration (Williams et al., 1991). Lattice-like structures characteristic of tubular myelin were not seen in mixtures containing SP-A and calcium but lacking SP-B (Williams et al., 1991). The effect of SP-A on surface activity also appears to be sensitive to the presence of SP-B. For example, Veldhuizen et al. (2000) reported that SP-A enhances the ability of SP-B to promote selective removal of non-DPPC lipids from monolayers on compression. SP-A alone did not enhance this refinement.

Submitted April 8, 2003, and accepted for publication June 18, 2003.

Address reprint requests to Dr. Kevin Keough, Dept. of Biochemistry, Memorial University of Newfoundland, St. John's, Newfoundland, A1B 3X9, Canada. Tel.: 709-737-2530; Fax: 709-737-2552; E-mail: kkeough@mun.ca or Dr. Michael Morrow, Dept. of Physics and Physical Oceanography, Memorial University of Newfoundland, St. John's, Newfoundland, A1B 3X7, Canada. Tel.: 709-737-4361; Fax: 709-737-8739; E-mail: myke@physics.mun.ca.

Abbreviations used: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; egg PG, phosphatidylglycerol made from egg yolk phosphatidylcholine; DPPC-d_{62}, 1,2-perdeuterodipalmitoyl-*sn*-glycero-3-phosphocholine; DPPC-d_4, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(α,β perdeutero)-choline.

0006-3495/03/10/2397/09 $2.00

Deuterium NMR has been used previously to examine the perturbation, by SP-B and SP-C, of bilayers containing chain-deuterated or headgroup-deuterated phospholipids (Simatos et al., 1990; Morrow et al., 1993; Dico et al., 1997). The surfactant proteins typically have little effect on phospholipid chain orientational order in the liquid-crystalline phase but do alter motions responsible for quadrupole echo decay in both the liquid-crystalline and gel bilayer phases.

In the presence of calcium, SP-A binds DPPC bilayers through the carbohydrate recognition domains (McCormack, 1998). SP-A does not bind to some other lipid species including dipalmitoylphosphatidylglycerol (McCormack, 1998). Because of its solubility in water, SP-A likely interacts with phospholipids at the bilayer surface rather than in the bilayer interior. Although calcium might be directly involved in the interaction between carbohydrate recognition domain and the bilayer surface, calcium binding does induce a conformational change in the SP-A carbohydrate recognition domain (Haagsman et al., 1990), which might activate a lipid binding site elsewhere on the carbohydrate recognition domain (McCormack, 1998).

The 18 carbohydrate recognition domains of the SP-A octadecamer form a cluster with a diameter of ~20 nm (Palaniyar et al., 1998). Tunneling electron micrographs presented by Palaniyar et al. (1998) show that calcium induces a more compact clustering of carbohydrate recognition domains in a given octadecamer and results in the octadecamer orienting so as to bring most or all of its carbohydrate recognition domains into contact with the lipid vesicle surface.

Because of its size, relative to individual phospholipids, the influence of a given SP-A complex might be expected to be distributed over more lipid molecules than is the case for individual hydrophobic surfactant proteins. The suggestion that the interaction between an individual carbohydrate recognition domain and the phospholipid at the bilayer interface is relatively weak and that the interaction of the bilayer with a given octadecamer is amplified by the simultaneous binding of several carbohydrate recognition domains (McCormack, 1998) is consistent with the images presented by Palaniyar et al. (1998).

Although the binding of SP-A to bilayers containing DPPC has been extensively investigated, little is known about the extent to which interaction with SP-A perturbs the properties of bilayers or the distribution of bilayer components in mixtures of DPPC and PG that are often used to model the lipid component of natural surfactant. In this work, ^{2}H NMR has been used to examine the extent to which SP-A interacts with liquid-crystalline and gel DPPC/egg-PG bilayers in the absence of other surfactant proteins. Its perturbation of chain orientational order and bilayer motion was examined by observation of spectra and measurement of quadrupole echo decay times in bilayers containing chain-deuterated DPPC-d_{62}. Its perturbation of headgroup orientation in the liquid-crystalline phase was inferred from measurements of headgroup-deuteron quadrupole splittings in bilayers containing choline-deuterated DPPC-d_4.

MATERIALS AND METHODS

Chain-perdeuterated DPPC-d_{62}, choline-deuterated DPPC-d_4 and egg-PG were obtained from Avanti Polar Lipids Inc. (Alabaster, AL) and were used without further purification.

Surfactant protein A was purified by injecting a suspension of porcine surfactant into 1-butanol and stirring for 30 min (Haagsman et al., 1987; Taneva et al., 1995). The mixture was then centrifuged at 10,000 × g for 20 min at 4°C. The supernatant was removed and the precipitate dried under N_2, after which it was washed twice in 20 ml of buffer (20 mM *n*-octyl-β-D-glucopyranoside, 100 mM NaCl, 10 mM HEPES, pH 7.4). After centrifugation twice at 100,000 × g for 30 min at 4°C, the resulting pellet was resuspended in a small volume of 5 mM HEPES, pH 7.4, and dialysed against 5 mM HEPES, pH 7.4, for 48 h. The sample was then centrifuged at 100,000 × g for 30 min at 4°C. The supernatant containing SP-A was stored in small aliquots at −20°C.

Concentrations of SP-A were estimated by the fluorescamine method using bovine serum albumin as a standard (Udenfriend et al., 1972). Lipid concentrations were determined by phosphorus analysis (Bartlett, 1959; Keough and Kariel, 1987).

To prepare samples, lipids were co-dissolved in chloroform/methanol(1:1, v/v). The solution was then dried under nitrogen, evacuated overnight in the presence of P_2O_5, and, following the example of Williams et al. (1991), hydrated in 10 ml of buffer (10 mM TRIS:145 mM NaCl:1 mM EDTA, pH 7.4) at 46°C for 1 h. The presence of EDTA in this buffer was intended to prevent interaction of lipids or SP-A with calcium before the controlled addition of excess calcium in the final step of the preparation. The lipid sample was then centrifuged at 50,000 × g for 30 min at 4°C. The pellet was resuspended in 1 ml of the supernatant from the centrifugation and the lipid concentration redetermined. For samples containing SP-A, the protein, in buffer, was added to give a final SP-A concentration in terms of total lipid and protein of 16% by weight. This corresponds to a ratio of ~250 lipids per SP-A monomer. After a 10-min incubation at 37°C, calcium was added to all samples to give a final concentration of 5 mM. After overnight incubation and mixing at 37°C, the samples were lightly compacted by centrifugation in a bench-top centrifuge at room temperature. The resulting pellet was then transferred to an NMR tube. Each sample contained ~10 mg of phospholipid of which ~7 mg was deuterium labeled. The total volume of each hydrated sample before final compaction was between 6 ml and 8 ml so that the incubation was carried out in a large excess of water and calcium relative to the lipid component in each sample.

Wideline deuterium NMR spectra and echo decay measurements were obtained with a locally constructed spectrometer using quadrupole echo pulse sequences (Davis et al., 1976). For all acquisitions, a repetition time of 0.5 s was used. Free induction decays used to obtain spectra of samples containing both DPPC-d_{62} and DPPC-d_4 were obtained in a 9.4 T superconducting solenoid using the quadrupole echo sequence with $\pi/2$ pulses of 5.0–5.5 μs separated by 50 μs (35 μs for the sample containing DPPC-d_{62}, egg-PG, and SP-A). Typically 20,000 transients were averaged for each spectrum. Signals were obtained using oversampling (Prosser et al., 1991) and digitized with effective dwell times of 4 μs for liquid crystal DPPC-d_{62} spectra, 2 μs for gel DPPC-d_{62} spectra, and 10 μs for DPPC-d_4 spectra. Spectrometer frequency and pulse lengths were adjusted before acquisition to minimize signal in the imaginary channel. To facilitate calculation of first moments, spectra were then symmetrized by zeroing residual noise in the imaginary channel. No line broadening was used. Signals were limited by the fact that each sample preparation began with no more than 7 mg of deuterated lipid. The narrow peak obtained in the center of most spectra likely arises from natural abundance deuterium in the water or from small particles generated during the preparation. Such features represented a negligible fraction of total spectral intensity and were not included in the calculation of first spectral moments.

Quadrupole splittings of DPPC-d_4 were obtained by using a ''dePakeing'' algorithm to transform the powder spectrum to the corresponding spectrum that would be obtained from an oriented sample (Bloom et al., 1981; Sternin et al., 1983; Whittall et al., 1989). Quadrupole echo decay measurements of samples containing DPPC-d_{62} were carried out at 3.5 T using $\pi/2$ pulses of 5.0 μs and up to 12,000 transients.

RESULTS

Fig. 1 shows ^{2}H NMR spectra at selected temperatures for DPPC-d_{62}/egg-PG (7:3) and DPPC-d_{62}/egg-PG (7:3) plus 16% SP-A by weight. Both samples were hydrated in buffer containing 5 mM Ca^{2+}. The SP-A concentration used here was chosen because this has been observed to be an amount that is conducive to the formation of tubular myelin-like structures in vitro (Suzuki et al., 1989; Williams et al., 1991) and because this concentration has also been used in this laboratory for other ^{2}H NMR studies for studies involving the formation of tubular myelin in vitro (M. Morrow, S. Harris, A. Dico, J. Stewart, S. Taneva, and K. Keough, unpublished).

At higher temperatures, the spectra in Fig. 1 are superpositions of Pake doublets characteristic of fast, axially symmetric CD bond reorientation in the liquid-crystalline phase. The splitting of a given doublet is proportional to the orientational order parameter, $S_{CD} = \langle 3\cos^2\theta - 1\rangle/2$ where θ is the angle between the CD bond and the axis, typically

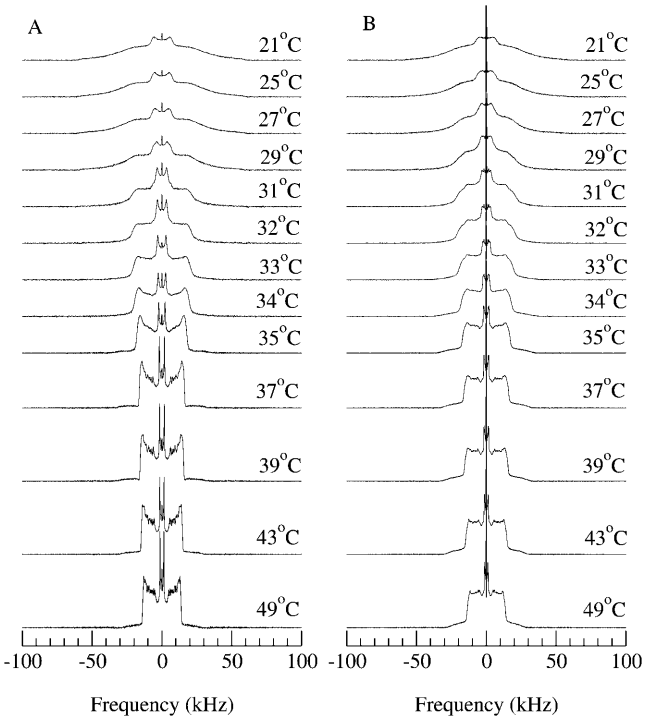

FIGURE 1 (*A*) Temperature dependence of selected ^{2}H NMR spectra for bilayers of DPPC-d_{62}/egg-PG (7:3) in buffer containing 5 mM Ca^{2+}. (*B*) Temperature dependence of selected ^{2}H NMR spectra for bilayers of DPPC-d_{62}/egg-PG (7:3) plus 16% SP-A by weight in buffer containing 5 mM Ca^{2+}.

the bilayer normal, about which the molecule is reorienting. The average is over motions of the CD bond that modulate the quadrupole interaction with correlation times that are short relative to the characteristic timescale ($\sim 10^{-5}$ s) of the deuterium NMR measurement (Davis, 1983). The distribution of splittings reflects the dependence of orientational order parameter on position, along the acyl chain, that characterizes the liquid-crystalline phase of saturated phospholipid bilayers. Comparison of dePaked spectra at 45°C (not shown) showed no evidence of a protein-induced local perturbation of the chain orientational order parameter profile. It should be noted, however, that the order parameter profile shape has been reported to be somewhat insensitive to local perturbations, likely due to the existence of a significant correlation length for changes in order parameter along the chain (Lafleur et al., 1990).

At low temperatures, the spectra are characteristic of the gel phase and reflect reorientation that is of intermediate rate and less axially symmetric on the timescale of the measurement. For both samples, the change from liquid crystal to gel proceeds continuously and none of the observed spectra displayed superpositions of liquid crystal and gel features that would be characteristic of two-phase coexistence.

In the liquid-crystalline phase, addition of SP-A has little effect on the range of doublet splittings in the DPPC-d_{62} spectrum but does affect the overall distribution of intensity across the spectrum. Doublet edges, arising from molecules reorienting about bilayer normals perpendicular to the applied field, are less prominent in the spectra of the sample containing SP-A and the distribution of intensity across the spectrum is flatter. This broadening of the lineshape is consistent with the observation of a protein-induced reduction in the liquid-crystalline quadrupole echo decay rate, as discussed below.

For a spectrum denoted by $f(\omega)$, the first spectral moment, defined as

$$M_1 = \frac{\int_0^\infty \omega f(\omega) d\omega}{\int_0^\infty f(\omega) d\omega}, \tag{1}$$

provides an indication of the average, over all deuterated segments, of the chain orientational order. Fig. 2 shows the temperature dependence of M_1 for the spectra of DPPC-d_{62}/egg-PG (7:3) and DPPC-d_{62}/egg-PG (7:3) plus 16% SP-A by weight. For temperatures above 36°C, the addition of SP-A reduces chain order only slightly. At lower temperatures, as spectra become increasingly characteristic of the gel phase, SP-A appears to interfere significantly with the development of chain orientational order.

The quadrupole echo pulse sequence consists of two $\pi/2$ pulses differing in phase by 90° and separated by an interval τ (Davis et al., 1976). Deuterons precessing after the initial $\pi/2$ pulse dephase due to heterogeneity of the orientation-dependent quadrupole interaction. Dephasing is reversed by

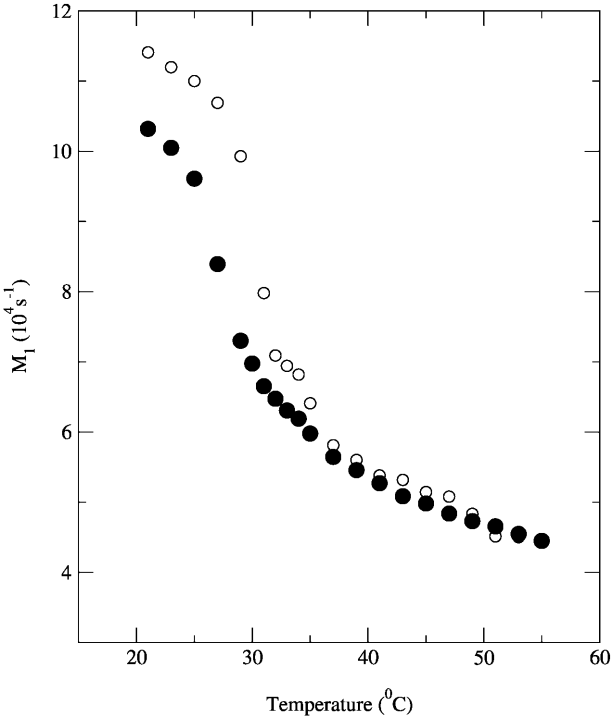

FIGURE 2 Temperature dependence of the first spectral moments for (○) DPPC-d_{62}/egg-PG (7:3) and (●) DPPC-d_{62}/egg-PG (7:3) plus 16% SP-A by weight. The uncertainty in determination of M_1 from a given spectrum is <0.5%. All samples are hydrated in buffer containing 5 mM Ca^{2+}.

the second $\pi/2$ pulse and an echo forms at time 2τ. The characteristic times for decay of the quadrupole echo can be obtained by varying τ and taking the initial slope from a semilogarithmic plot of echo amplitude versus the time at which each echo is formed.

Motions that change the quadrupole interaction while an echo is being formed contribute to echo decay. For motions having short correlation times (τ_c), relative to the inverse spectral width, the contribution to the echo decay rate is proportional to τ_c (Abragam, 1961). For slower motions, the contribution to the echo decay rate is proportional to τ_c^{-1} (Pauls et al., 1985). In the liquid-crystalline phase, slow diffusive or collective motions and faster local motions can both contribute to echo decay (Bloom and Sternin, 1987; Bloom and Evans, 1991; Stohrer et al., 1991). As bilayers are cooled into more ordered phases, the correlation times for lateral diffusion and collective motions increase and their contributions to echo decay decrease.

The correlation times for more local motions, such as rotation about the bilayer normal or rotation of chain segments about bonds, also increase with decreasing temperature. Their contributions to echo decay rate can thus pass through a maximum and then decrease with increasing correlation time. Typically, the resulting quadrupole echo decay time is only weakly dependent on temperature in the liquid-crystalline phase, where contributions from slow and fast motions compete. On cooling the sample, the echo decay time generally passes through a minimum, as the bilayer begins to order, and then increases as remaining motions continue to slow.

Fig. 3 shows echo decay times at selected temperatures for DPPC-d_{62}/egg-PG (7:3) and DPPC-d_{62}/egg-PG (7:3) plus 16% SP-A by weight. Above 36°C, the echo decay times for both samples do not vary significantly with temperature. Addition of SP-A increases the quadrupole echo decay rate significantly as reflected by the reduced values for T_{2e}. In contrast, SP-A has little effect on orientational order of the chain deuterons in the liquid-crystalline phase. Orientational order parameters reflect averaging of the quadrupole interaction on timescales that are short relative to the characteristic time ($\sim 10^{-5}$ s) for the NMR observation. It is thus likely that the observed effect of SP-A on quadrupole echo decay in the liquid-crystalline phase reflects perturbation of longer range diffusive or collective motions rather than the faster local motions reflected by the orientational order parameters for each segment.

At lower temperatures, for which the spectra are increasingly characteristic of the gel phase, SP-A reduces the echo decay time only slightly. This is very different from the effects of the hydrophobic pulmonary surfactant proteins SP-B and SP-C, which are both observed to significantly reduce the rate at which echo decay time increases with decreasing temperature in the gel phase (Simatos et al., 1990; Dico et al., 1997).

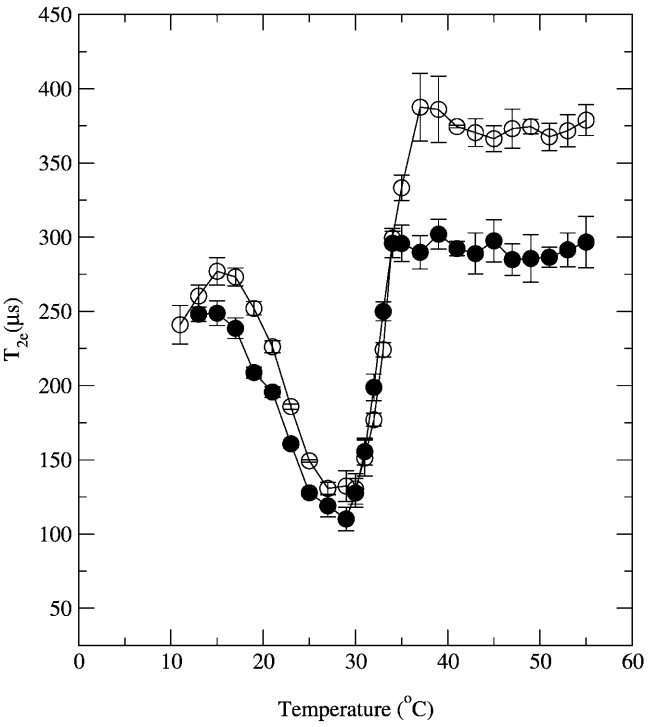

FIGURE 3 The temperature dependence of quadrupole echo decay times for (○) DPPC-d_{62}/egg-PG (7:3) and (●) DPPC-d_{62}/egg-PG (7:3) plus 16% SP-A by weight, both in buffer containing 5 mM Ca^{2+}.

The appearance of a local maximum in echo decay time at lower temperatures has been previously observed in bilayers containing lipids with phosphatidylglycerol headgroups (Dico et al., 1997). Because the quadrupole echo decay rate is inversely proportional to correlation time (τ_c) in the limit of long τ_c for any motion, the echo decay time must ultimately increase with decreasing temperature. The observation of a local maximum at temperatures for which the spectra are characteristic of gel phase behavior indicates that for this class of samples, there must be some motion with a relatively short correlation time that persists into the gel phase. The nature of this motion, and the reason why it is more apparent in the presence of negatively charged PG bilayer components remain to be addressed. In the context of the current results, it is interesting that in the gel phase the presence of SP-A has little effect on motions, including any reflected by the local T_{2e} maximum, that contribute to quadrupole echo decay.

In the phosphatidylcholine headgroup, the methylene bonded to the oxygen of the phosphate and the adjacent methylene are labeled as α and β, respectively. Counter-directional shifts in the quadrupole splittings of the α- and β-deuterons have been attributed to changes in the orientation of the phosphatidlycholine headgroup with respect to the bilayer normal (Sixl and Watts, 1983; Seelig et al., 1987; Scherer and Seelig, 1989). Incorporation of charged amphiphiles into a bilayer can influence the torque applied to the phosphatidylcholine headgroup electric dipole without substantially perturbing bilayer packing (Seelig et al., 1987; Scherer and Seelig, 1989). Addition of negative surface charge, which would be expected to tilt the phosphatidylcholine headgroup toward the bilayer surface by attraction of the $N^+(CH_3)_3$ group, was found to increase the α-deuteron quadrupole splitting and reduce the β-deuteron splitting (Scherer and Seelig, 1989; Macdonald et al., 1991). The opposite effect was observed with the incorporation of positive amphiphiles into the bilayer (Scherer and Seelig, 1989).

Addition of some amphiphiles such as sterols (Habiger et al., 1992) or the lung surfactant protein SP-C (Morrow et al., 1993) can give rise to responses of the α- and β-deuteron splittings that differ significantly in magnitude. This may reflect competition between the electrostatic effects on headgroup orientation, which would tend to shift the α- and β-splittings in opposite directions, and changes in the amplitude of headgroup reorientation due to altered lipid packing or steric effects, which might tend to shift the splittings of all headgroup deuterons in the same direction.

Fig. 4 shows spectra at selected temperatures for DPPC-d_{62}/egg-PG (7:3) and DPPC-d_{62}/egg-PG (7:3) plus 16% SP-A by weight. Both samples were hydrated in buffer containing 5 mM Ca^{2+}. The central spike in each spectrum likely arises from natural abundance deuterium in the buffer. Its prominence reflects the relatively weak signal from the small number of choline deuterons in these samples.

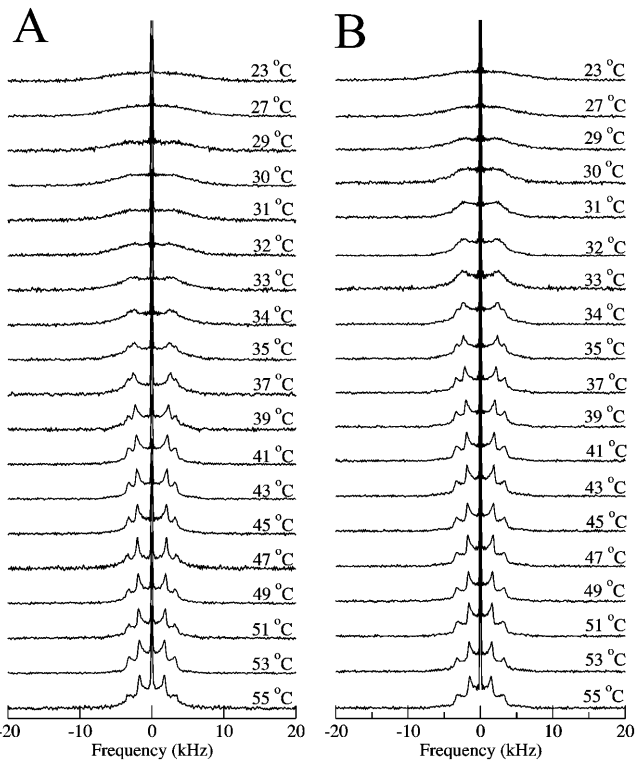

FIGURE 4 (*A*) The temperature dependence of ^2H NMR spectra for bilayers of DPPC-d_4/egg-PG (7:3) in buffer containing 5 mM Ca^{2+}. (*B*) The temperature dependence of ^2H NMR spectra for bilayers of DPPC-d_4/egg-PG (7:3) plus 16% SP-A by weight in buffer containing 5 mM Ca^{2+}.

Relative to the situation in a pure DPPC bilayer, interaction of negatively charged egg-PG headgroups with the $N^+(CH_3)_3$ group of the phosphatidylcholine in these samples results in the headgroup orientation shifting, on average, away from the bilayer normal. Based on the expectation that this would reduce the quadrupole splitting of the β-deuteron doublet and increase that of the α-deuteron doublet, relative to their values in pure headgroup-deuterated DPPC, the smaller splittings in Fig. 4 can be assigned to the β-deuterons. Quadrupole splittings for α- and β-headgroup deuterons in the liquid-crystalline phases of both samples were obtained by transforming each powder spectrum, using a ''dePakeing'' algorithm (Bloom et al., 1981; Sternin et al., 1983; Whittall et al., 1989) to obtain the spectrum that would be observed for an oriented bilayer sample. Splittings obtained in this way are plotted against temperature in Fig. 5.

For both samples, the β-deuteron splittings decrease with increasing temperature. The α-splitting does not change significantly with temperature. This likely reflects the combined effects of temperature-dependent changes in headgroup orientation and in the amplitude of headgroup reorientation. Acyl chain orientational order decreases with increasing temperature. If the resulting increase in area per lipid relaxes constraints on headgroup orientation and allows the headgroup average orientation to lie more parallel to the bilayer surface, the expected result would be a decrease in

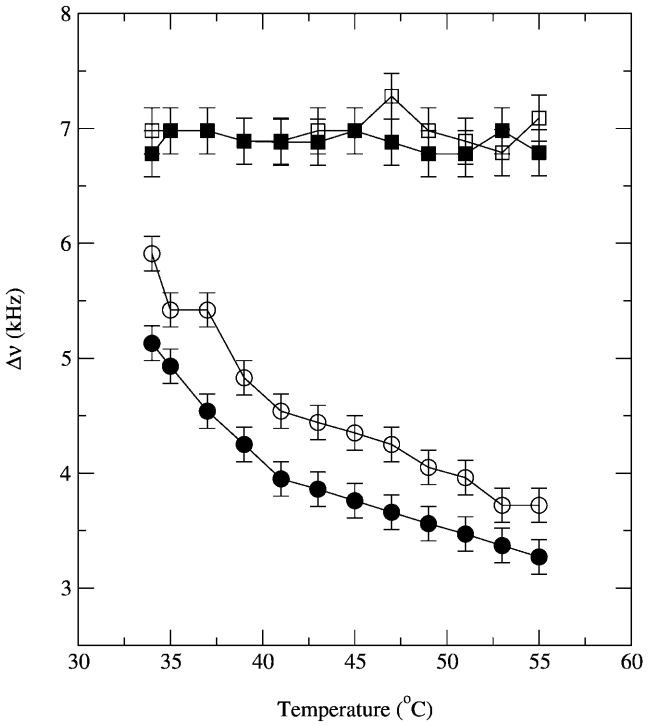

FIGURE 5 Temperature dependence of deuteron quadrupole splittings for the α (□) and β (○) choline deuterons in DPPC-d_4/egg-PG (7:3) and for α (■) and β (●) choline deuterons in DPPC-d_4/egg-PG (7:3) plus 16% SP-A by weight. Both samples are hydrated in buffer containing 5 mM Ca^{2+}.

β-headgroup deuteron splittings and an increase in α-headgroup deuteron splittings. A simultaneous increase in the amplitude of headgroup reorientation about the average orientation would tend to reduce the splittings for all headgroup deuterons. Both effects could combine to reduce the β-splitting but would tend to cancel for the α-deuteron. Situations in which deuterons on one headgroup segment respond more strongly than others to changes in temperature or other parameters have been reported previously (Gally et al., 1975; Bonev and Morrow, 1995).

At a given temperature in the liquid-crystalline range, addition of SP-A reduces the β-deuteron splitting slightly but, like changes in temperature, has little effect on α-deuteron splitting. By analogy to the observed response to changes in temperature, it appears that the interaction of SP-A with the bilayer shifts the average phosphatidylcholine headgroup orientation away from the bilayer normal while also increasing the amplitude of reorientation slightly.

DISCUSSION

The results reported here imply an interaction of SP-A with DPPC/egg-PG bilayers that is strong enough to observably influence bilayer properties but that is also qualitatively different from the effect of hydrophobic pulmonary surfactant proteins SP-B and SP-C.

In the liquid-crystalline phase, SP-A affects headgroup orientation and motions that contribute to quadrupole echo decay but has little effect on chain order. The headgroup deuteron splittings observed in the DPPC-d_4/egg-PG sample without SP-A present reflect the effect of the anionic PG headgroup on orientation of the phosphatidylcholine headgroup. The α-deuteron splitting is higher and the β-deuteron splitting lower than would be seen for DPPC-d_4 at the same temperature in the absence of PG (Morrow et al., 1993). This is consistent with the torque exerted on the phosphatidylcholine headgroup electric dipole by the addition of negatively charged PG headgroups to the bilayer surface. The observation that addition of SP-A does not remove the effect of PG, and indeed reduces the β-deuteron splitting slightly further, suggests that SP-A does not significantly alter the lateral distribution of DPPC and egg-PG in the bilayer.

The observation that the DPPC headgroup β-deuteron splitting decreases with temperature whereas the α-splitting remains approximately constant has been interpreted as resulting from a change in average torsional angle about the C_α-C_β bond due to increased torsional oscillations around this bond (Gally et al., 1975). It was concluded that the average separation between the choline methyl group and the headgroup phosphorus decreased with increasing temperature. In general, however, raising the temperature will increase the amplitude of overall headgroup reorientation as well as reorientation about the C_α-C_β bond. This would be expected to reduce both α- and β-headgroup deuteron splittings in contrast to what is observed. Raising the temperature also decreases chain order and increases area available to the headgroup, thus accommodating an average headgroup orientation further from the bilayer normal. Such a change in average orientation, with its implied counter-directional shifts in α- and β-headgroup deuteron splittings (Seelig et al., 1987; Scherer and Seelig, 1989; Macdonald et al., 1991), may account for the overall insensitivity of α-splitting to temperature. It should be noted that such an effect does not preclude a change in average headgroup conformation as suggested by Gally et al. (1975) due to increased oscillation about the C_α-C_β bond. It is likely that observed temperature dependence of DPPC headgroup deuteron splittings reflects a combination of changes in headgroup tilt, changes in average headgroup conformation, and changes in the amplitude of overall and internal headgroup reorientation.

A rough estimate of the extent to which interaction with SP-A alters headgroup interaction can be obtained by noting that the change in β-headgroup deuteron splitting resulting from addition of SP-A at a given temperature is comparable in magnitude to the change resulting from an increase in temperature of 5–10°C. In analogy with the expected effect of temperature, this likely involves a slight rotation of the average headgroup orientation away from the bilayer normal along with increased amplitude of overall headgroup reorientation and reorientation about the C_α-C_β bond which,

as suggested by Gally et al. (1975) may imply a change in average headgroup conformation. It is interesting that addition of SP-A does not significantly alter the rates with which deuteron splittings change with temperature. This suggests that interaction with the protein has little effect on the average relative orientations of the α- and β-choline segments. The observation that chain orientational order in the liquid-crystalline phase is not significantly reduced by the presence of SP-A suggests that any change in headgroup orientation or conformation is small enough to be accommodated without a significant increase in overall area per lipid.

The headgroup spectrum in the gel phase does not show well-defined doublet edges and cannot be interpreted in terms of headgroup orientation in the same way as in the liquid-crystalline phase. The effect of SP-A on chain orientational order, as indicated by first spectral moments, does suggest that the interaction of SP-A with the bilayer surface constrains the packing of lipid molecules in the more ordered phase. Any tendency for SP-A to tilt the headgroup away from the bilayer normal, as suggested for the liquid-crystalline phase, likely interferes with the reduction in headgroup area required to accommodate increased chain orientational order in the gel phase.

In the liquid-crystalline phase, quadrupole echo decay is sensitive to changes in motions that range from fast rotations about bonds and the bilayer normal to slower motions, like diffusion and collective bilayer motions and undulations, that involve longer length scales (Bloom and Sternin, 1987; Bloom and Evans, 1991; Stohrer et al., 1991). Motions with correlation times that are short relative to the inverse spectral width ($\sim 10^{-5}$ s) can contribute to motional narrowing (Bloom and Sternin, 1987).

The presence of SP-A significantly perturbs quadrupole echo decay rates for chain deuterons in the liquid-crystalline phase but has little effect on chain orientational order. The interaction of SP-A with the bilayer thus appears to influence longer range diffusive or collective motions but not local rotations that would be expected to be fast and contribute to motional narrowing in the liquid-crystalline phase. Even though the hydrophobic surfactant proteins SP-B and SP-C are expected to interact more strongly with the bilayer interior, their effects on liquid-crystalline chain order and quadrupole echo decay are qualitatively similar to what is reported here for SP-A, which is relatively hydrophilic and presumably does not interact as directly with the bilayer interior.

The effect of SP-A on quadrupole echo decay at lower temperatures is qualitatively different from that of SP-B or SP-C. Motions that are slow in the liquid-crystalline phase will be even slower at low temperature and are not expected to contribute significantly to quadrupole echo decay as the bilayer becomes more ordered. Rotations about the bilayer normal, or about individual bonds, that are fast in the liquid-crystalline phase will slow, on cooling, into the regime where their contributions to the echo decay rate are inversely proportional to their correlation times. Quadrupole echo decay times in the gel phase are thus generally expected to increase with decreasing temperature.

As temperature is reduced in the gel phase, the hydrophobic surfactant proteins SP-B and SP-C appear to interfere with the slowing of motions that contribute to quadrupole echo decay in the gel phase (Simatos et al., 1990; Dico et al., 1997). The result is that gel phase echo decay times are reduced by the presence of SP-B and SP-C. SP-A has little effect on the quadrupole echo decay in the gel phase suggesting that its interaction with the bilayer primarily perturbs motions, such as bilayer undulations and collective modes, that contribute to echo decay in the liquid-crystalline phase but might be expected to have less effect on quadrupole echo decay in the gel phase. This is consistent with SP-A being outside of the membrane and interacting primarily at the bilayer surface.

As noted above, the observation of a local maximum in quadrupole echo decay time versus temperature below the liquid crystal to gel transition implies the persistence, into the gel phase, of some motion that remains fast enough for its contribution to the echo decay rate to be proportional to its correlation time. Although the nature of that motion cannot be fully characterized by the observations reported here, it also appears not to be significantly perturbed by the presence of SP-A.

Although the specific contribution of SP-A to pulmonary surfactant function is not fully understood, SP-A has been shown to affect the physical properties and behavior of surfactant material in a number of ways. The observation of tubular myelin in vitro has been found to require the presence of both SP-A and SP-B (Suzuki et al., 1989; Williams et al., 1991; Poulain et al., 1992). SP-A, particularly in combination with SP-B, has been found to influence the morphology of DPPC/egg-PG monolayers (Taneva and Keough, 2000) and to promote selective adsorption of DPPC from subphase vesicles into a monolayer via the formation of structures resembling tubular myelin (Veldhuizen et al., 2000). In studies of porcine lung surfactant lipid extract monolayers, SP-A, in the presence of calcium, is observed to be dispersed in the fluid phase and aggregated at the boundaries of condensed domains (Worthman et al., 2000).

It is important to note that the fraction of lipids directly bound by an SP-A carbohydrate recognition domain is likely to be much smaller than the fraction of lipids influenced by the proximity of SP-A to the membrane surface and that the perturbations reported here are averages over the population of deuterium-labeled DPPC in each sample. Despite this constraint, these observations provide some insight into the nature of the interaction between SP-A and the phospholipid bilayer and, in particular, point to the interaction of SP-A with the bilayer as being peripheral. This is consistent with indications, from binding studies and electron microscopy, that SP-A octadecamers interact with phopholipid bilayer surfaces through the cluster of carbohydrate recognition domains (McCormack, 1998; Palaniyar et al., 1998).

Reports that SP-A binds DPPC and dipalmitoylglycerol but not DPPG, palmitic acid or lysophosphatidylcholine have been interpreted as indicating that the interaction between SP-A and phospholipids requires the lipid to have two chains linked to a glycerol backbone (McCormack, 1998). This suggests that the interaction between SP-A occurs near the hydrophobic-hydrophilic interface of the bilayer and that the interaction is constrained by lipid packing. The observation here that SP-A perturbs the DPPC headgroups but has little effect on orientational order of the chains in the liquid-crystalline phase is consistent with the expectation that SP-A associates with the headgroup region of the bilayer. The effect of SP-A on chain order in the gel phase suggests that the localization of SP-A on the membrane surface persists into the gel phase and that the resulting constraint on headgroup orientation interferes with the reduction in area per lipid on cooling.

It is significant that the headgroup deuteron spectra in the liquid-crystalline phase display only a single pair of splittings in the presence of SP-A. This indicates that over the timescale of the NMR experiment ($\sim 10^{-5}$ s), the perturbation due to SP-A is uniformly distributed over all of the observed headgroups. This suggests that a large fraction of the highly mobile liquid-crystalline lipids are influenced in a dynamic, and possibly nonspecific, way by the large carbohydrate recognition domain clusters of bound SPA octadecamers. If, in addition, a small fraction of the lipid population is tightly bound to SP-A, the signal from these lipids might not be observed due to spectral broadening or low abundance. The evidence for a uniform lipid environment provided by headgroup deuteron spectra in the presence of SP-A also suggests that interaction with SP-A does not significantly alter the distribution of lipid components in the bilayer.

Hydrophobic proteins, including SP-B and SP-C, significantly perturb motions responsible for quadrupole echo decay in the gel phase (Simatos et al., 1990; Dico et al., 1997). The absence of a significant perturbation of quadrupole echo decay in the gel phase, where echo decay is likely dominated by local lipid reorientation rather than large scale bilayer undulations, is also consistent with a primarily peripheral interaction of SP-A with the bilayer. The possibility that the effect of SP-A on quadrupole echo decay times in the liquid-crystalline phase is primarily due to perturbation of collective motions like bilayer undulations is consistent with electron micrographs of similarly prepared DPPC/PG/SP-A bilayers presented by Williams et al. (1991). Those images show multilamellar structures with bilayers uniformly separated by ~20 nm. The spaces between adjacent bilayers contain particles that are interpreted as being SP-A octadecamers. The perturbation of interbilayer interactions by intercalation of such large particles into the multilamellar structure would likely have a stronger effect on collective bilayer undulations than on the motions of individual lipids and chain segments within the local bilayer frame of reference.

From images of samples prepared for electron microscopy, SP-A in the presence of calcium is known to interact with the bilayer surface through the carbohydrate recognition domains of the octadecamer (Williams et al., 1991; Palaniyar et al., 1998). The work reported here has shown a significant perturbation of hydrated DPPC/egg-PG bilayer dynamics by SP-A. These observations suggest that the interaction of SP-A with hydrated bilayers is also focused at the bilayer surface and that the perturbation, on the timescale of the NMR experiment, is distributed uniformly over the DPPC population of the mixed bilayer. Persistence of the interaction into the gel phase is also demonstrated.

The issue of whether the prime role of SP-A involves host defense or surfactant function remains controversial (McCormack, 1998). The perturbation of bilayer properties by SP-A reported here suggest that if the role of SP-A is primarily host defense, its interaction with lipids must presumably be accommodated within any mechanism responsible for maintaining surfactant function.

K. M. W. Keough is Chief Scientist, Health Canada, Ottawa, Ontario, Canada.

This research was supported by grants from the Natural Science and Engineering Research Council of Canada to M.R.M. and from the Canadian Institutes of Health Research to K.M.W.K.

REFERENCES

Abragam, A. 1961. The Principles of Nuclear Magnetism. Oxford University Press, London.

Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* 234:466–468.

Benson, B. J., M. C. Williams, K. Sueishi, J. Goerke, and T. Sargeant. 1984. Role of calcium ions in the structure and function of pulmonary surfactant. *Biochim. Biophys. Acta.* 793:18–27.

Bloom, M., J. H. Davis, and A. L. MacKay. 1981. Direct determination of the oriented sample NMR spectrum from the powder spectrum for systems with local axial symmetry. *Chem. Phys. Lett.* 80:198–202.

Bloom, M., and E. Evans. 1991. Observation of surface undulations on the mesoscopic length scale by NMR. *In* Biologically Inspired Physics. L. Peliti, editor. Plenum Press, New York. 137–147.

Bloom, M., and E. Sternin. 1987. Transverse nuclear spin relaxation in phospholipid bilayer membranes. *Biochemistry.* 26:2101–2105.

Bonev, B. B., and M. R. Morrow. 1995. Hydrostatic pressure-induced conformational changes in phosphatidylcholine headgroups: a ^{2}H NMR study. *Biophys. J.* 69:518–523.

Curstedt, T., J. Johansson, P. Persson, A. Eklund, B. Robertson, B. Löwenadler, and H. Jörnvall. 1990. Hydrophobic surfactant-associated polypeptides: SP-C is a lipopeptide with two palmitoylated cysteine residues, whereas SP-B lacks covalently linked fatty acyl groups. *Proc. Natl. Acad. Sci. USA.* 87:2985–2989.

Davis, J. H., K. R. Jeffrey, M. Bloom, M. I. Valic, and T. P. Higgs. 1976. Quadrupole echo deuteron magnetic resonance spectroscopy in ordered hydrocarbon chains. *Chem. Phys. Lett.* 42:390–394.

Davis, J. H. 1983. The description of membrane lipid conformation, order and dynamics by ^{2}H-NMR. *Biochim. Biophys. Acta.* 737:117–171.

Dico, A. S., J. Hancock, M. R. Morrow, J. Stewart, S. Harris, and K. M. W. Keough. 1997. Pulmonary surfactant protein SP-B interacts similarly with dipalmitoylphosphatidylglycerol and dipalmitoylphosphatidylcholine in phosphatidylcholine/phosphatidylglycerol mixture. *Biochemistry.* 36:4172–4177.

Drickamer, K., M. S. Dordal, and L. Reynolds. 1986. Mannose-binding proteins isolated from rat liver contain carbohydrate-recognition domains linked to collagenous tails. Complete primary structures and homology with pulmonary surfactant apoprotein. *J. Biol. Chem.* 261:6878–6887.

Gally, H.-U., W. Niederberger, and J. Seelig. 1975. Conformation and motion of the choline head group in bilayers of dipalmitoyl-3-*sn*-phosphatidylcholine. *Biochemistry.* 14:3647–3652.

Gil, J., and O. K. Reiss. 1973. Isolation and characterization of lamellar bodies and tubular myelin from rat lung homogenates. *J. Cell Biol.* 58:152–171.

Goerke, J. 1998. Pulmonary surfactant: functions and molecular composition. *Biochim. Biophys. Acta.* 1408:79–89.

Haagsman, H. P., T. Sargeant, P. V. Hauschka, B. J. Benson, and S. Hawgood. 1990. Binding of calcium to SP-A, a surfactant-associated protein. *Biochemistry.* 29:8894–8900.

Haagsman, H. P., S. Hawgood, T. Sargeant, D. Buckley, R. T. White, K. Drikamer, and B. J. Benson. 1987. The major lung surfactant protein, SP 28–36, is a calcium dependent, carbohydrate-binding protein. *J. Biol. Chem.* 262:13877–13880.

Habiger, R. G. K., J. M. Cassal, H. J. M. Kempen, and J. Seelig. 1992. Influence of stigmastanol and stigmastanyl-phosphorylcholine, two plasma cholesterol lowering substances, on synthetic phospholipids membranes. A ^{2}H- and ^{31}P-NMR study. *Biochim. Biophys. Acta.* 1103:69–76.

Hawgood, S., H. Efrati, J. Schilling, and B. J. Benson. 1985. Chemical characterization of lung surfactant apoproteins: amino acid composition, N-terminal sequence and enzymic digestion. *Biochem. Soc. Trans.* 13:1092–1096.

Hawgood, S., M. Derrick, and F. Poulain. 1998. Structure and properties of surfactant protein B. *Biochim. Biophys. Acta.* 1408:150–160.

Johansson, J. 1998. Structure and properties of surfactant protein C. *Biochim. Biophys. Acta.* 1408:161–172.

Keough, K. M. W., and N. Kariel. 1987. Differential scanning calorimetric studies of aqueous dispersions of phosphatidylcholines containing two polyenoic chains. *Biochim. Biophys. Acta.* 902:11–18.

King, R. J., and J. A. Clements. 1972. Surface active material from dog lung. I. Method of isolation. *Am. J. Physiol.* 223:707–714.

Lafleur, M., P. R. Cullis, and M. Bloom. 1990. Modulation of the orientational order profile of the lipid acyl chain in the L_α phase. *Eur. Biophys. J.* 19:55–62.

Macdonald, P. M., J. Leisen, and F. M. Marassi. 1991. Response of phosphatidylcholine in the gel and liquid-crystalline states to membrane surface charges. *Biochemistry.* 30:3558–3566.

McCormack, F. X. 1998. Structure, processing and properties of surfactant protein A. *Biochim. Biophys. Acta.* 1408:109–131.

Morrow, M. R., S. Taneva, G. A. Simatos, L. A. Allwood, and K. M. W. Keough. 1993. ^{2}H NMR studies of the effect of pulmonary surfactant SP-C on the 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine headgroup: a model for transbilayer peptides in surfactant and biological membranes. *Biochemistry.* 32:11338–11344.

Palaniyar, N., R. A. Ridsdale, C. F. Holterman, K. Inchley, F. Possmayer, and G. Harauz. 1998. Structural changes of surfactant protein A induced by cations reorient the protein on lipid bilayers. *J. Struct. Biol.* 122:297–310.

Pauls, K. P., A. L. MacKay, O. Söderman, M. Bloom, A. K. Tangea, and R. S. Hodges. 1985. Dynamic properties of the backbone of an integral membrane polypeptide measured by ^{2}H-NMR. *Eur. Biophys. J.* 12:1–11.

Pérez-Gil, J., and K. M. W. Keough. 1998. Interfacial properties of surfactant proteins. *Biochim. Biophys. Acta.* 1408:203–217.

Poulain, F. R., L. Allen, M. C. Williams, R. L. Hamilton, and S. Hawgood. 1992. Effects of surfactant apolipoproteins on liposome structure: implications for tubular myelin formation. *Am. J. Physiol.* 262:L730–L739.

Prosser, R. S., J. H. Davis, F. W. Dahlquist, and M. A. Lindorfer. 1991. ^{2}H nuclear magnetic resonance of the gramicidin A backbone in a phospholipid bilayer. *Biochemistry.* 30:4687–4696.

Putman, E., L. A. J. M. Creuwels, L. M. G. van Golde, and H. P. Haagsman. 1996. Surface properties, morphology and protein composition of pulmonary surfactant subtypes. *Biochem. J.* 320:599–605.

Sanders, D. L., R. J. Hassett, and A. E. Vatter. 1980. Isolation of lung lamellar bodies and their conversion to tubular myelin figures in vitro. *Anat. Rec.* 198:485–501.

Scherer, P. G., and J. Seelig. 1989. Electric charge effects on phospholipids headgroups. Phosphatidylcholine in mixtures with cationic and anionic amphiphiles. *Biochemistry.* 28:7720–7728.

Seelig, J., P. M. Macdonald, and P. G. Scherer. 1987. Phospholipid head groups as sensors of electric charge in membranes. 1987. *Biochemistry.* 26:7535–7541.

Sen, A., S.-W. Hui, M. Mosgrober-Anthony, B. A. Holm, and E. A. Egan. 1988. Localization of lipid exchange sites between bulk lung surfactants and surface monolayer: freeze fracture study. *J. Colloid Interface Sci.* 126:355–360.

Simatos, G. A., K. B. Forward, M. R. Morrow, and K. M. W. Keough. 1990. Interaction between perdeuterated bimyristoylphosphatidylcholine and low molecular weight pulmonary surfactant protein SP-C. *Biochemistry.* 29:5807–5814.

Sixl, F., and A. Watts. 1983. Headgroup interactions in mixed phopholipid bilayers. *Proc. Natl. Acad. Sci. USA.* 80:1613–1615.

Sternin, E., M. Bloom, and A. L. MacKay. 1983. De-Pake-ing of NMR spectra. *J. Magn. Reson.* 55:274–282.

Stohrer, J., G. Gröbner, D. Reimer, K. Weisz, C. Mayer, and G. Kothe. 1991. Collective lipid motions in bilayer membranes studied by transverse deuteron spin relaxation. *J. Chem. Phys.* 95:672–678.

Suzuki, Y., Y. Fujita, and K. Kogishi. 1989. Reconstitution of tubular myelin from synthetic lipids and proteins associated with pig pulmonary surfactant. *Am. Rev. Respir. Dis.* 140:75–81.

Taneva, S., and K. M. W. Keough. 2000. Differential effects of surfactant protein A on regional organization of monolayers containing surfactant protein B or C. *Biophys. J.* 79:2010–2023.

Taneva, S., T. McEachern, J. Stewart, and K. M. W. Keough. 1995. Pulmonary surfactant protein SP-A with phospholipids in spread monolayers at the air-water interface. *Biochemistry.* 34:10279–10289.

Udenfriend, S., S. Stein, P. Bohlen, W. Dairman, W. Loimgrukes, and M. Weigele. 1972. Fluorescamine: a reagent for assay of amino acids, peptides, and primary amines in the picomole range. *Science.* 178:871–872.

Veldhuizen, R., K. Nag, S. Argeig, and F. Possmayer. 1998. The role of lipids in pulmonary surfactant. *Biochim. Biophys. Acta.* 1408:90–108.

Veldhuizen, E. J. A., J. J. Batenburg, L. M. G. van Golde, and H. P. Haagsman. 2000. The role of surfactant proteins in DPPC enrichment of surface films. *Biophys. J.* 79:3164–3171.

Von Neergaard, K. 1929. Neue Auffassungen über einen Grundebeguff der Atemmechancik. Die Tetrakionskraft der Lunge, Abhängig von des Oberflächenspannung in den Alveolen. *Z. Gesamte Exp. Med.* 66:373–394.

Voss, T., H. Eistetter, K. P. Schäfer, and J. Engel. 1988. Macromolecular organization of natural and recombinant lung surfactant protein SP 28–36. Structural homology with the complement factor C1q. *J. Mol. Biol.* 201:219–227.

Whittall, K. P., E. Sternin, M. Bloom, and A. L. MacKay. 1989. Time- and frequency-domain ''DePakeing'' using inverse theory. *J. Magn. Reson.* 84:64–71.

Williams, M. C., S. Hawgood, and R. L. Hamilton. 1991. Changes in lipid structure produced by surfactant proteins SP-A, SP-B, and SP-C. *Am. J. Respir. Cell Mol. Biol.* 5:41–50.

Worthman, L.-A. D., K. Nag, N. Rich, M. L. F. Ruano, C. Casals, J. Pérez-Gil, and K. M. W. Keough. 2000. Pulmonary surfactant protein A interacts with gel-like regions in monolayers of pulmonary surfactant lipid extract. *Biophys. J.* 79:2657–2666.

Yu, S. H., N. Smith, P. G. R. Harding, and F. Possmayer. 1983. Bovine pulmonary surfactant: chemical composition and physical properties. *Lipids.* 18:522–529.