Identification of Phosphocholine Plasmalogen as a Lipid Component in Mammalian Pulmonary Surfactant Using High-Resolution ^{31}P NMR Spectroscopy†

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ABSTRACT: High-resolution ^{31}P NMR spectroscopy was used to analyze the phospholipid composition of mammalian pulmonary surfactant from two different sources. Under conditions which considerably narrow the usually broad ^{31}P phospholipid signals, solution-phase NMR spectra of these surfactant preparations unequivocally demonstrate that a phosphocholine plasmalogen (i.e., 1-*O*-(1′-alkenyl)-2-acyl-*sn*-glycero-3-phosphocholine) exists as a major secondary component (~4 mol %) in mammalian pulmonary surfactant. Phosphocholine (PC) plasmalogen was identified in preparations obtained from both adult cow lung surfactant extract as well as in ovine (lamb) fetal pulmonary liquid. PC plasmalogens have not previously been identified any mammalian pulmonary surfactant preparation. The amount of PC plasmalogen in these preparations occurs at fractional levels that are comparable to that of phosphoglycerol (PG), which previously had been thought of as the second-most common phospholipid class in pulmonary surfactant. The presence of PC plasmalogen in pulmonary surfactant may have important physiological ramifications and immediately suggests new directions for biochemical and biophysical investigations of pulmonary surfactant.

Pulmonary surfactant is a complex mixture of lipids (90 wt %), proteins (8 wt %), and carbohydrates (2 wt %) which is synthesized and secreted by alveolar epithelial type II cells. The function of this heterogeneous surfactant mixture is to reduce the surface tension at the air–alveolar interface in the lungs to near zero, thereby providing the necessary mechanical stability to the broncho-alveolar units required for breathing (Scarpelli, 1988). Inadequate synthesis and release of pulmonary surfactant is responsible for the respiratory distress experienced by premature infants as well as some forms of adult respiratory distress syndrome. Greater than 85% of the lipids found in pulmonary surfactant are phospholipids (King, 1984). To date, phosphocholines and phosphoglycerols (PC's and PG's)[1] have been identified as the primary phospholipid classes which are present in mammalian pulmonary surfactant. PC's comprise roughly 70–85% of pulmonary surfactant by weight, with the specific PC species 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) making up more than 60% of the total PC component. The class of lipids containing PG and their headgroup may comprise up to 10% of the pulmonary surfactant phospholipids. The other phospholipid classes (PE, SM, PI, PS, and CL) occur at relatively low levels [<2–5 mol % (King, 1984)]. At least four proteins, termed SP-A, SP-B, SP-C, and SP-D, have also been isolated from pulmonary surfactant (Ng et al., 1983; Hagwood et al., 1987; Curstedt et al., 1988; Johansson et al., 1988; Persson et al., 1990; Hawgood & Schiffer, 1991). The specific functional roles of these surfactant proteins have just recently begun to be addressed (Pastrana et al., 1991).

Due to the in vivo spreading properties of the final, functional form of the pulmonary surfactant which contributes to lowering the surface tension in the alveolus, it has been postulated that the most physiologically relevant configuration of pulmonary surfactant is that of a monomolecular lipid–protein film at the air–alveolar lining (King & Clements, 1972; Goerke & Clements, 1986), although this theory has never been definitively proven and other configurations are probable (Scarpelli, 1988). Because of the presence of long saturated hydrocarbon chains, DPPC molecules can pack tightly to form a condensed film and remain absorbed at the air–water interface when subjected to large compressive forces even at physiological temperatures (37 °C). These unique physical properties of the DPPC monolayer may explain its presence at high levels in mammalian pulmonary surfactant. In kinetic terms, however, DPPC alone spreads poorly at the air–water interface, and it has been postulated that secondary surfactant components, such as Ca^{2+}, PG, unsaturated lipids, or proteins, may be needed to facilitate the transfer of DPPC from the hypophase to the air–water interface (Hagwood et al., 1985, 1987). In recent years, biophysical approaches have been used to study both the native pulmonary surfactant in addition to well-defined model phospholipid and surfactant protein mixtures in order to develop a molecular-level understanding of the role of these additional components in pulmonary surfactant function (King & Clements, 1972; Hook et al., 1984; Keough et al., 1985; Mautone et al., 1987; Shiffer et al., 1988; Dluhy et al., 1989; Reilly et al., 1989; Simatos et al., 1990; Baatz et al., 1990, 1991; Salmon & Wiedmann, 1991; Pastrana et al., 1991). These studies have provided important insights into surfactant structure and function that could ultimately lead to the rational development of effective

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[1] Abbreviations: CL, cardiolipin; CLSE, cow lung surfactant extract; EDTA, (ethylenedinitrilo)tetraacetic acid; FID, free induction decay; FPL, fetal pulmonary liquid; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; PC plasmalogen, 1-*O*-(1′-alkenyl)-2-acyl-*sn*-glycero-3-phosphocholine; PE, phosphoethanolamine; PG, phosphoglycerol; PI, phosphoinositol; RDS, respiratory distress syndrome; PS, phosphoserine; SM, sphingomyelin; TLC, thin-layer chromatography.

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replacement surfactants for the treatment of RDS. Clearly, a comprehensive knowledge of pulmonary surfactant composition is necessary to guide these biophysical studies.

The composition of the phospholipid classes in isolated pulmonary surfactant preparations has typically been determined using two-dimensional TLC followed by recovery of the phosphorus-visualized spots. Quantitative inorganic phosphorus analysis followed by gas–liquid chromatography of the transesterified fatty acid methyl esters is then used to identify the lipid headgroup classes and acyl chain fatty acid distribution (Kates, 1972; Lau & Keough, 1981). Attempts have also been made to develop HPLC methods to separate and quantitate the phospholipids found in pulmonary surfactant (Dethoff et al., 1986; Bonanno et al., 1992). This approach has not achieved the widespread use of the TLC methods, primarily due to selective column retention of certain lipid classes as well as the difficulty of quantitating PG components in the surfactant mixture. In addition, the surfactant phospholipid composition as determined by HPLC differs from the composition determined by other, well-established, standard methods. In particular, these HPLC methods (Dethoff et al., 1986; Bonanno et al., 1992) appear to overestimate the levels of PE and PG in pulmonary surfactant at the expense of PC (King, 1984).

While chromatography has previously been the primary analytical tool for determining the composition of phospholipid mixtures, it has recently been shown that high-resolution ^{31}P NMR spectroscopy may also be utilized for the multicomponent analysis of phospholipids in mixtures (Meneses & Glonek, 1988). This assay relies on an NMR line-narrowing reagent which allows the baseline resolution of most of the phospholipid classes based on headgroup structure over a very narrow chemical shift range. In addition, derivatives of these phospholipid classes may also be resolved. The effectiveness of this technique has been illustrated by measuring the phospholipid profiles of tissue extracts (Meneses & Glonek, 1988). Rana et al. (1991) have also used this method to determine the phospholipid composition of the outer membranes from wild-type and LPS mutant strains of *Salmonella typhimurium*.

Recent work in this laboratory has used this high-resolution ^{31}P NMR method to analyze binary mixtures of synthetic model pulmonary surfactant phospholipids (Rana et al., 1993a,b). In this paper, we present ^{31}P NMR spectroscopic evidence which demonstrates unequivocally the existence of a plasmalogen derivative of phosphocholine in natural preparations of mammalian surfactant from adult cow lung surfactant extract (CLSE) as well as ovine (lamb) fetal pulmonary liquid (FPL). Plasmalogens are a variation of the usual diacylglycerophospholipid in which the *sn*-1 chain exists in a 1-*O*-(1′-alkenyl) ether linkage while the *sn*-2 chain remains in the carbonyl ester form. This is the first literature report of the existence of PC plasmalogen lipids in mammalian pulmonary surfactant. We find that PC plasmalogen occurs at fractional levels that are comparable to PG, which previously had been thought of as the second-most common phospholipid class in surfactants. We also discuss possible reasons why the presence of this phospholipid has previously gone undetected in surfactant preparations. The presence of PC plasmalogen in pulmonary surfactant may have important physiological ramifications and immediately suggests new directions for biochemical and biophysical investigations of pulmonary surfactant.

EXPERIMENTAL PROCEDURES

Materials. Phospholipid standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. Semisynthetic PC plasmalogen with palmitic acid synthetically incorporated in the *sn*-2 position was obtained as a custom synthesis from Avanti Polar Lipids. Bovine heart PC with known PC plasmalogen contamination (Pugh et al., 1977) was purchased from Sigma Chemical Co. (St. Louis, MO) as a 10 mg mL^{-1} $CHCl_3$ solution. All other chemicals used were of reagent grade.

Isolation of CLSE and Mature FPL. The cow lung surfactant extract (CLSE) was isolated using routine procedures (Hall et al., 1992) as follows. The lungs of freshly killed calves were lavaged with 150 mM NaCl. The lavagate was first centrifuged at 300*g* for 10 min to remove cells and debris prior to centrifuging the remaining supernatant at 12000*g*. These centrifugation steps remove the possibility of contamination from red blood cell components in the final preparation. The hydrophobic extract of the resulting pellet was isolated by the method of Bligh and Dyer (1959). This extract was then dried under a N_2 stream.

The ovine fetal pulmonary liquid (FPL) was obtained directly from the lungs of a mature, 147-day gestation lamb fetus in utero before the first breath was taken in a bloodless preparation. The FPL was centrifuged at 300*g* for 10 min to remove cells and debris. The hydrophobic extract of the FPL was obtained by a Bligh–Dyer extraction and subsequently dried under N_2.

Sample Preparation for ^{31}P NMR Spectroscopy. Stock solutions of the individual synthetic phospholipids were prepared by dissolving 5–10 mg of lipid in 5 mL of $CHCl_3$. The exact phospholipid concentrations were determined by an inorganic phosphorus assay (Chen et al., 1956). Phospholipid standards were prepared for ^{31}P NMR spectroscopy by taking aliquots of the synthetic lipid stock solutions, evaporating the organic solvent with a gentle stream of Ar, and then dissolving the resulting dry phospholipid film in 0.6 mL of $CDCl_3$ and 1.2 mL of the NMR line-narrowing reagent. Samples of the hydrophobic extracts of bovine lung (CLSE) and ovine FPL were prepared for NMR spectroscopy by dissolving approximately 15–20 mg of dried film in 1 mL of $CDCl_3$ and 2 mL of the line-narrowing reagent. The solutions prepared by this method were biphasic; as much of the upper aqueous layer as possible was removed by aspiration. The samples were adjusted in the NMR tube turbine so that only the $CDCl_3$ phase was in the transmitter/receiver coil.

The potassium and cesium line-narrowing reagents were prepared as previously outlined (Meneses & Glonek, 1988) with modifications described by Rana et al. (1991). Briefly, the procedure for preparation of the line-narrowing reagent involved titrating a 0.2 M suspension of the free acid form of EDTA with either KOH or CsOH until the pH was 6.00. The solution was lyophilized, and the salt was dissolved in enough H_2O to yield a 0.2 M solution of EDTA. Four volumes of freshly distilled methanol was then added. Additional water was added if the line-narrowing reagent appeared cloudy due to any undissolved salt.

^{31}P NMR Spectroscopy. ^{31}P NMR spectra were obtained on a Bruker AMX 400 NMR spectrometer operated at 161.977 MHz with a 5-mm probe at ambient temperature. The field frequency was locked to $CDCl_3$. Typically, 1000–2000 transients were collected for the phospholipid standards, and 16 000 transients were collected for the CLSE and ovine FPL samples. In both cases, a sweep width of 1639.34 Hz (10.121 ppm), a flip angle of 45°, and a acquisition time of 1.25 s were

employed. Broad-band proton decoupling was employed using the WALTZ16 pulse sequence. The decoupler was gated on only during data acquisition in order to suppress NOE effects. The T_1 values of the phospholipid signals were approximately 1.6 s. An interpulse delay of 2 s was used during spectral acquisition, which was found to be sufficient to allow the nuclei to completely relax while minimizing the interpulse delay time (Rana et al., 1993b). Before computation of the Fourier transform, the FID's were multiplied by an exponential window function with the line-broadening exponent set at a value of 1. The digital resolution was 0.80 Hz per data point and typical line widths at half-height were about 1.6 Hz.

The ^{31}P NMR signals in the bovine CLSE and ovine FPL spectra were assigned by comparison with spectra of phospholipid standards. Chemical shift values are reported relative to 85% phosphoric acid. The NMR signal due to 1,2-diacyl-PC was used as an internal standard at a chemical shift value of –0.84 ppm. The chemical shift values we observed are, in general, in very good agreement with previously published values (Meneses & Glonek, 1988); however, the chemical shift values for the acidic phospholipids PI and PG in the CLSE and FPL preparations were found to differ slightly. We attribute these differences to the fact that this NMR assay is highly sensitive not only to the nature of the counterion in the line-narrowing reagent but also to the ionic character of the initial sample. The chemical shift differences we observe in the PI and PG peaks may be due to the presence of different types of cationic contaminants in the CLSE and FPL preparations which are not completely removed by the line-narrowing reagent. These chemical shift differences were only observed for acidic phospholipid headgroups, not zwitterions. After data collection, the NMR spectral data files were downloaded from the Bruker NMR spectrometer to a desktop personal computer for further data analysis. Integrated areas of the component NMR peaks were obtained using the Lab-Calc software package (Galactic Industries Corporation, Salem, NH). The integrated areas of overlapped NMR peaks were determined with the Lab-Calc curve fitting routine by using a Lorentzian line shape and fixing the chemical shift values and the number of component bands.

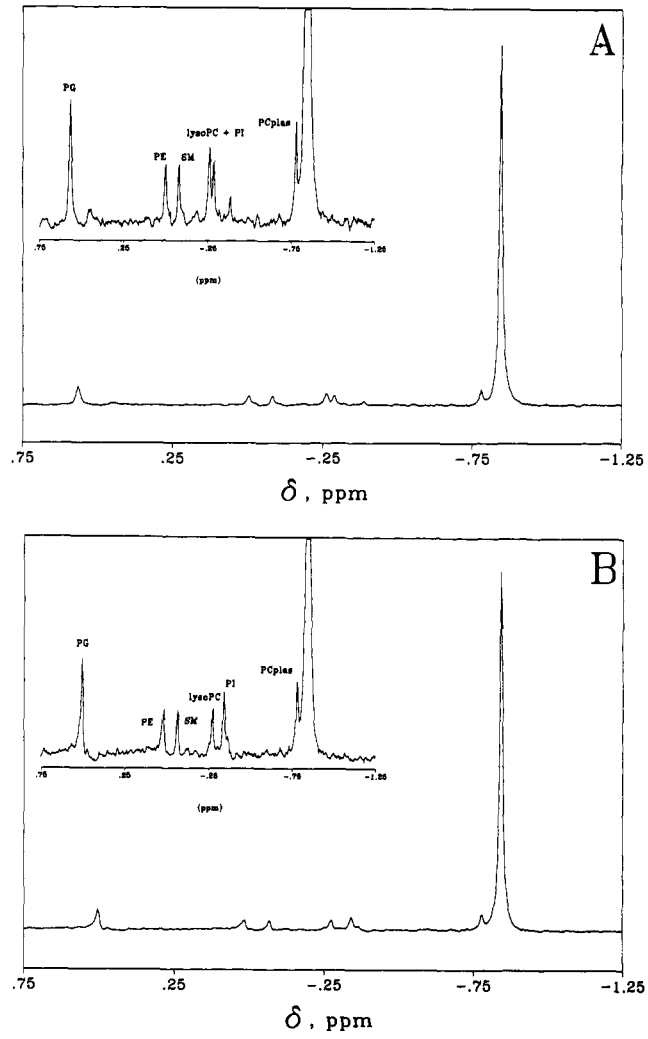

FIGURE 1: High-resolution ^{31}P NMR spectra of cow lung surfactant extract (CLSE) dissolved in the K^+ (A) and Cs^+ (B) line-narrowing reagent. The insets show the same spectra with an expanded y-axis. The peak labels are as follows: PG, phosphoglycerol; PE, phosphoethanolamine; SM, sphingomyelin; lyso-PC: 1-lyso-2-acyl-*sn*-glycero-3-phosphocholine; PI, phosphoinositol; plasPC, PC plasmalogen.

RESULTS AND DISCUSSION

The high-resolution ^{31}P NMR method described here and in previous reports has the ability to separate a wide variety of phospholipid classes on the basis of headgroup structure over a narrow chemial shift range (Meneses & Glonek, 1988; Rana et al., 1991). In addition, this method is able to quantitatively detect the fractional composition of different phospholipid classes in a complex mixture (Rana et al., 1991, 1993a,b). We have used the properties of this ^{31}P NMR assay to perform a quantitative multicomponent analysis of the phospholipid composition in pulmonary lung surfactant preparations obtained from the two different mammalian sources.

Figure 1 panels A and B show high-resolution ^{31}P NMR spectra of CLSE dissolved in the potassium and cesium line-narrowing reagents, respectively. The most prominent feature in both spectra is the intense PC peak which is assigned a value of –0.84 ppm in accordance with the work of Meneses and Glonek (1988). The signals at 0.57, 0.01, and –0.08 ppm in the spectrum of CLSE dissolved in the potassium line-narrowing reagent arise from PG, PE, and SM, respectively. The two signals between –0.26 and –0.29 ppm are due to lyso-PC and PI. However, the unambiguous assignment of these two peaks is not possible, since the separation between these two NMR lines is of the same order as the uncertainty in the chemical shift, the precision of which is estimated to be ±0.015 ppm (Meneses & Glonek, 1988). We observe a chemical shift precision that is very close to this value for samples dissolved in the potassium line-narrowing reagent. Upfield changes in the chemical shift values of ^{31}P NMR signals of acidic phospholipids have been observed by substituting the K^+ counterion with Cs^+ in the line-narrowing reagent (Meneses & Glonek, 1988). The chemical shift values of the ^{31}P NMR lines of zwitterionic phospholipids are insensitive to the counterion. This effect was used to unequivocally assign the PI and lyso-PC signals by measuring the high-resolution ^{31}P NMR spectrum of CLSE in the presence of the Cs^+ line-narrowing reagent. As can be seen from Figure 1B, the chemical shift values of the zwitterionic components are unaffected by replacing K^+ with Cs^+ in the line-narrowing reagent, whereas the PG and PI signals are shifted from values of 0.57 and –0.26 ppm to 0.50 and –0.34 ppm, respectively. The minor components CL, PS, and PA were not detected in this CLSE preparation.

Another signal present in the NMR spectrum of the CLSE preparation is the peak at –0.78 ppm which exists as a shoulder next to the large diacyl-PC signal at –0.84 ppm. We have

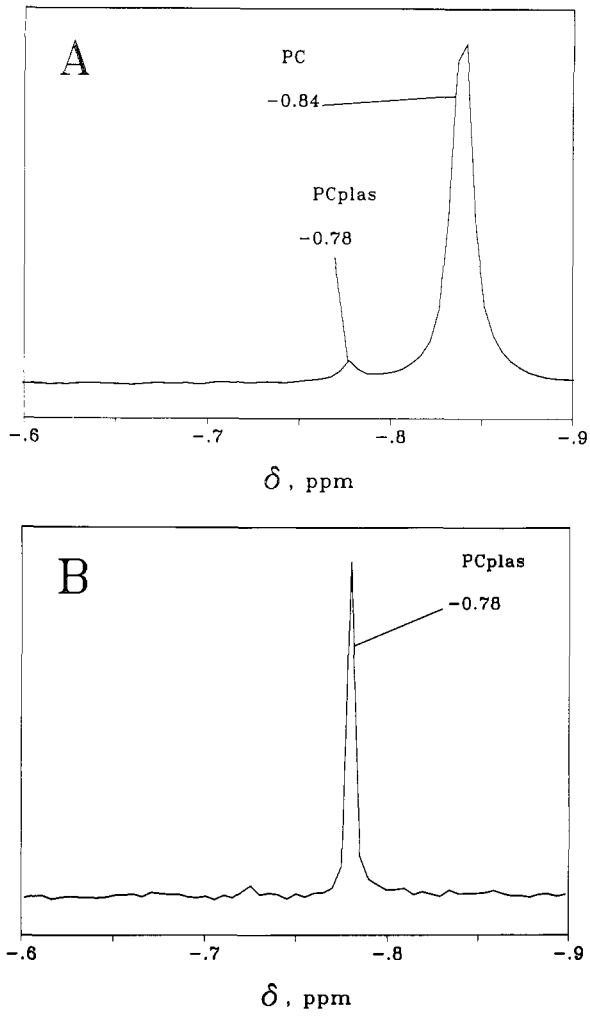

FIGURE 2: High-resolution ^{31}P NMR spectrum of phospholipid standards used to identify the –0.78 ppm signal. (A) Bovine heart PC with known PC plasmalogen concentration dissolved in the K^+ line-narrowing reagent. The major 1,2-diacyl-PC NMR peak occurs at –0.84 ppm; a smaller PC plasmalogen peak occurs shifted 0.06 ppm downfield at –0.78 ppm. (B) Semisynthetic PC plasmalogen with 16:0 palmitic acid incorporated into the *sn*-2 position. The major signal at –0.78 ppm is due to the PC plasmalogen linkage.

been able to identify this signal at –0.78 ppm as arising from a PC plasmalogen derivative. It has previously been reported using this NMR assay that a 1-*O*-(1′-alkenyl) ether linkage in the *sn*-1 position of a phospholipid plasmalogen causes an approximate 0.07 ppm downfield shift relative to the parent PE or PC phospholipid (Meneses & Glonek, 1988). We have confirmed the assignment of the –0.78 ppm signal as originating from a PC plasmalogen by two different methods. In the first method, we obtained the high-resolution ^{31}P NMR spectrum of bovine heart PC (Figure 2A), which is known to contain PC plasmalogen contamination (Pugh et al., 1977). Only two peaks were observable in the ^{31}P NMR spectrum of the bovine heart PC dissolved in the K^+ line-narrowing reagent. Figure 2A shows a large signal at –0.84 ppm which is readily identified as the diacyl-PC signal. In addition, a much smaller peak is observed in this spectrum at –0.78 ppm, which corresponds to the PC plasmalogen contamination.

The second method used to identify the –0.78 ppm signal as originating from PC plasmalogen used a semisynthetic PC plasmalogen in which the *sn*-2 acyl chain was enriched with 16:0 palmitic acid. Figure 2B shows the ^{31}P NMR spectrum of the semisynthetic PC plasmalogen in the K^+ line-narrowing

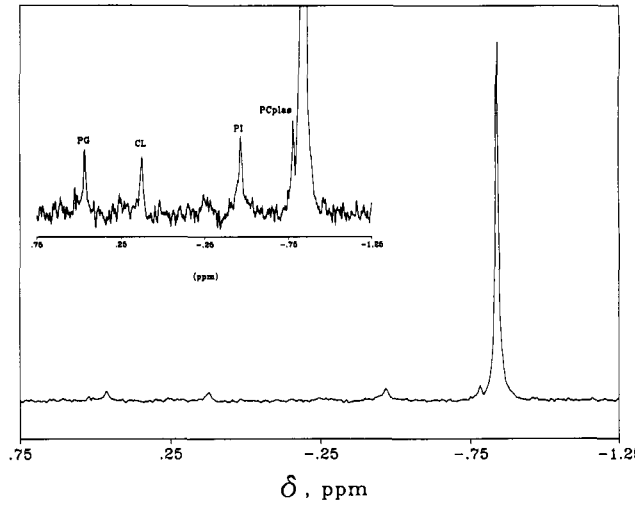

FIGURE 3: High-resolution ^{31}P NMR spectra of ovine fetal pulmonary liquid (FPL) dissolved in the Cs^+ line-narrowing reagent. The inset shows the same spectrum with an expanded *y*-axis. The peak labels are as follows: PG, phosphoglycerol; CL, cardiolipin; PI, phosphoinositol; plasPC, PC plasmalogen.

reagent. The major signal in this spectrum arises at –0.78 ppm, confirming the assignment of this peak to PC plasmalogen.

On the basis of the assignment of the –0.78 signal to PC plasmalogen, we obtained the fractional percentage of the individual phospholipid classes present in the CLSE preparation from integration of the individual peak areas. The mole percent of each phospholipid component in the CLSE was found to be PC, 82%; PC plasmalogen, 4%; PI, 3%; lyso-PC, 2%; SM, 2%; PE, 2%; and PC, 5%.

We have also determined the phospholipid profile of ovine FPL obtained in utero from a mature, 147-day gestation, lamb fetus. Figure 3 shows the high-resolution ^{31}P NMR spectrum of ovine FPL dissolved in the Cs^+ line-narrowing reagent. As with CLSE, PC is the most dominant phospholipid component in ovine FPL, comprising 87 mol % of the phospholipids. The presence of PC plasmalogen in ovine FPL is readily apparent from the NMR spectrum. Using the same methodology described above, we found that the PC plasmalogen phospholipid occurs at a level of 4 mol % in ovine FPL. In addition, PI, CL, and PG are identified at levels of 4, 3, and 2 mol %, respectively.

These data use high-resolution ^{31}P NMR spectroscopy to identify PC plasmalogen as an important secondary component of pulmonary surfactant. The presence of PC plasmalogen in two different mammalian surfactant preparations (i.e., CLSE from adult cows and in the FPL from fetal lambs) argues that this phospholipid may be found universally in mammalian pulmonary surfactant. In these preparations, PC plasmalogen is found at levels that are comparable to PG, which had previously been regarded as one of the two major phospholipid classes found in pulmonary surfactant (King, 1984). Therefore, the presence of this lipid class may play an important role in pulmonary surfactant physiology.

Although the composition of pulmonary surfactant from a wide variety of sources has been exhaustively characterized, it is not surprising that PC plasmalogen has not been previously identified as a component of pulmonary surfactant. The most widely used method of determining the phospholipid composition of tissue extracts and biological membranes is to separate the phospholipids by TLC, recover the resolved phospholipids from the TLC plate after visualization, and

quantitate the level of each individual phospholipid using a colorimetric assay for inorganic phosphorus (Kates, 1972). Unfortunately, this method of analysis will not detect plasmalogen components, since intact plasmalogens cannot be separated from the corresponding diacyl parent compounds by chromatographic methods (Horrocks & Sharma, 1982). Separation of PC and PE plasmalogen from diacyl-PC and -PE requires the removal or modification of the polar headgroup by enzymatic or chemical means. Further purification of these plasmalogens requires the selective degradation of the diacylphospholipids by alkaline or enzymatic hydrolysis using either phospholipase D or A_2 which exploits the lesser reactivity of plasmalogens (Horrocks & Sharma, 1982).

The high-resolution ^{31}P NMR technique developed by Meneses and Glonek (1988) offers significant advantages for characterizing the phospholipid composition of biological fluids, tissue extracts, and biological membranes compared to the standard chromatographic approaches. The most obvious advantage of the NMR method is the ability to perform multicomponent analyses on phospholipid mixtures without the need for prior separation of the individual components. The high-resolution ^{31}P NMR method is able to resolve all major classes of phospholipids and their lyso- and plasmalogen derivatives and is insensitive to potential chromatographic artifacts such as the incomplete extraction of phospholipids from a TLC plate or the selective retention of phospholipids on a HPLC column. Since each phospholipid molecule contains a single phosphorus atom, the integrated NMR intensity is proportional to the number of phospholipid molecules of that particular class present in the mixture and is not influenced by other structural features of the phospholipid molecule. The only limitation of using this ^{31}P NMR spectroscopic method to quantitate the composition of phospholipid mixtures is the inherent insensitivity of the NMR experiment. We find that the practical limit of detection for this technique is about 25–50 μg of phospholipid. However, it may be possible to increase the sensitivity of this experiment using other NMR techniques (e.g., polarization transfer). We are currently investigating these possibilities.

The results of this report highlight the importance of characterizing the effect of the 1-*O*-(1′-alkenyl) linkage in the *sn*-1 position of PC plasmalogens on the phase properties, structure, and spreading dynamics of model phospholipid monolayers at the air–water interface. These biophysical studies should help to establish the functional significance of PC plasmalogen in pulmonary surfactant and may provide new insight into the mechanism of pulmonary surfactant physiology.

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