Membrane Insertion and Lipid-Protein Interactions of Bovine Seminal Plasma Protein PDC-109 Investigated by Spin-Label Electron Spin Resonance Spectroscopy

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ABSTRACT The interaction of the major acidic bovine seminal plasma protein, PDC-109, with dimyristoylphosphatidylcholine (DMPC) membranes has been investigated by spin-label electron spin resonance spectroscopy. Studies employing phosphatidylcholine spin labels, bearing the spin labels at different positions along the *sn*-2 acyl chain indicate that the protein penetrates into the hydrophobic interior of the membrane and interacts with the lipid acyl chains up to the 14th C atom. Binding of PDC-109 at high protein/lipid ratios (PDC-109:DMPC = 1:2, w/w) results in a considerable decrease in the chain segmental mobility of the lipid as seen by spin-label electron spin resonance spectroscopy. A further interesting new observation is that, at high concentrations, PDC-109 is capable of (partially) solubilizing DMPC bilayers. The selectivity of PDC-109 in its interaction with membrane lipids was investigated by using different spin-labeled phospholipid and steroid probes in the DMPC host membrane. These studies indicate that the protein exhibits highest selectivity for the choline phospholipids phosphatidylcholine and sphingomyelin under physiological conditions of pH and ionic strength. The selectivity for different lipids is in the following order: phosphatidylcholine $\approx$ sphingomyelin $\geq$ phosphatidic acid (pH 6.0) $>$ phosphatidylglycerol $\approx$ phosphatidylserine $\approx$ androstanol $>$ phosphatidylethanolamine $\geq$ *N*-acyl phosphatidylethanolamine $\gg$ cholestane. Thus, the lipids bearing the phosphocholine moiety in the headgroup are clearly the lipids most strongly recognized by PDC-109. However, these studies demonstrate that this protein also recognizes other lipids such as phosphatidylglycerol and the sterol androstanol, albeit with somewhat reduced affinity.

INTRODUCTION

The seminal plasma of mammals contains a group of acidic proteins that bind to the spermatozoa. These proteins in the bovine seminal plasma have been purified and are biochemically well characterized. There are four proteins, which are named BSP-A1, BSP-A2, BSP-A3, and BSP-30-kDa proteins (Manjunath and Sairam, 1987; Manjunath et al., 1987). The first three are roughly of the same molecular mass (between 13 and 16 kDa), whereas the molecular mass of the BSP-30-kDa protein, as the name implies, is ~30,000 Da (Manjunath et al., 1987). BSP-A1 and BSP-A2 have the same primary structure and differ only in extent of glycosylation, and their mixture is also referred to as PDC-109 (Esch et al., 1983). Collectively, these four proteins are referred to as bovine seminal plasma proteins, or BSP proteins (Desnoyers and Manjunath, 1992). In this study, the mixture of BSP-A1 and BSP-A2 has been used without separating the glycosylated and unglycosylated forms of the protein, and therefore the term PDC-109 will be used to refer to it.

Biochemical studies have shown that the interaction of BSP proteins with spermatozoa is mediated by their interaction with specific phospholipids, in particular with the zwitterionic phospholipid, phosphatidylcholine (Desnoyers and Manjunath, 1992). The interaction of PDC-109 with spermatozoa leads to the efflux of choline phospholipids and cholesterol from the sperm plasma membrane (referred to as cholesterol efflux), which appears to be an important step in the capacitation process (Thérien et al., 1998; Moreau et al., 1999). The single polypeptide chain of PDC-109 consists of two tandemly repeating fibronectin type II domains, each of which contains a choline phospholipid binding site, and both the binding sites are necessary for inducing lipid efflux (Esch et al., 1983; Desnoyers and Manjunath, 1992, 1993; Moreau et al., 1998). Because capacitation is a necessary event before fertilization can occur, it is important to understand the interaction of this protein with sperm cell membranes to understand the molecular events involved in the capacitation process. Most importantly, such an understanding potentially can lead to the development of novel anti-fertility drugs.

Not only from the biological point of view, but also from a biophysical perspective, the BSP proteins are of great interest. Phosphatidylcholines (or the sphingolipid analog,

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Abbreviations used: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; SM, sphingomyelin; ESR, electron spin resonance; *n*-PCSL, 1-acyl-2-[*n*-(4, 4-dimethyloxazolidine-*N*-oxyl)]stearoyl-*sn*-glycero-3-phosphocholine; 14-PGSL, -PESL, -PSSL, and -PASL, 1-acyl-2-[14-(4, 4-dimethyloxazolidine-*N*-oxyl)]stearoyl-*sn*-glycero-3-phosphoglycerol, -phosphoethanolamine, -phosphoserine, and -phosphoric acid; 14-SMSL, *N*-[14-(4, 4-dimethyloxazolidine-*N*-oxyl)stearoyl]-sphingosine-1-phosphocholine; 14-NAPESL, 1,2-diacyl-*sn*-glycero-3-(*N*-14-(4, 4-dimethyloxazolidine-*N*′-oxyl) stearoyl)phosphoethanolamine; CSL, 4′,4′-dimethylspiro(5α-cholestane-3,2′-oxazolidin)-3′-yloxyl; ASL, 17β-hydroxy-4′,4′-dimethylspiro(5α-androstane-3,2′-oxazolidin)-3′-yloxyl; MLV, multilamellar vesicle; FnII, fibronectin type-II.

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sphingomyelin) are the major phospholipid component of mammalian cell membranes. It is generally assumed that these zwitterionic phospholipids play a passive structural role as the major building blocks in biological membranes, without any specific interactions. Our own results on lipid-protein interactions with both integral and peripheral membrane proteins generally indicate a lack of specific interactions with phosphatidylcholine (reviewed in Marsh and Horváth, 1998). In view of the ubiquitous nature of phosphatidylcholines (or sphingomyelin) in mammalian membranes, the identification of proteins that specifically interact with these particular phospholipids is of great interest and potential importance. This is not solely from a structural and functional standpoint, but also with regard to the possibility that pathologies might occur arising from specific protein or peptide interactions with this major membrane constituent.

In the work described here, we have characterized the interaction of PDC-109 with phosphatidylcholine membranes in detail by using spin-label ESR spectroscopy. We have particularly concentrated on the influence of the protein on the lipid chain mobility and on the membrane chain-melting transition, on the penetration of the protein into the membrane and its direct interaction with the lipid chains, and on the specificity of interaction with particular lipid species by using spin-labeled lipids with different polar headgroups.

MATERIALS AND METHODS

Materials

Phosphorylcholine chloride (Ca^{2+} salt), choline chloride, and Tris base were from Sigma (St. Louis, MO). Sephadex G-50 (superfine), DEAE Sephadex A-25, and DEAE Sephadex A-50 were obtained from Pharmacia Biotech (Uppsala, Sweden). DMPC was obtained from Avanti Polar Lipids (Alabaster, AL). The spin-labeled fatty acids and phosphatidylcholines (*n*-PCSLs) were synthesized according to procedures outlined in Marsh and Watts (1982). Spin-labeled phospholipids with different polar headgroups (14-PGSL, 14-PESL, and 14-PSSL) were prepared from spin-labeled phosphatidylcholine (14-PCSL) by phospholipase D-catalyzed headgroup exchange, as described in the same reference. Spin-labeled *N*-acyl phosphatidylethanolamine, 14-NAPESL, with the spin label in the *N*-acyl chain was synthesized as described by Swamy et al. (2000). Spin-labeled sphingomyelin (14-SMSL) was prepared as described by Hoffmann et al. (2000). Cholestane spin label (CSL) and androstanol spin label (ASL) were obtained from Syva (Palo Alto, CA).

Samples of bovine semen and extraction of BSP proteins

Samples of bovine semen, freshly collected from Ongole bulls with the aid of an artificial vagina, were kindly provided by the Department of Animal Reproduction, Acharya N. G. Ranga University of Agricultural Sciences, Hyderabad, and Lam Farm, Department of Animal Breeding of the same university at Guntur, Andhra Pradesh, India. The samples were stored on ice, for a maximum of 9 h (until they were brought to the laboratory) and then centrifuged at 3000 rpm in a refrigerated centrifuge to separate sperm cells and seminal plasma. Total proteins from the seminal plasma were precipitated by adding cold ethanol, and the precipitated proteins were dissolved in distilled water and lyophilized. The lyophilized protein fraction was delipidated by extraction with a mixture of *n*-butanol/di-isopropylether (40/60; v/v), as described by Desnoyers and Manjunath (1993).

Purification of PDC-109

PDC-109 was purified by a modification of the procedure reported by Calvete et al. (1996). In the modified procedure, delipidated BSP protein fraction obtained according to the procedure of Desnoyers and Manjunath (1992, 1993) was used instead of the bovine seminal plasma used by Calvete et al. (1996). The delipidated BSP protein fraction was dissolved in 50 mM Tris buffer, 0.15 M NaCl, 5 mM EDTA, pH 7.4 (TBS-I) and subjected to gel filtration on a column of Sephadex G-50 superfine (2.5 × 170 cm), preequilibrated with 50 mM Tris, 1.0 M NaCl, 5 mM EDTA, pH 6.4 (TBS-II). The major peak containing PDC-109 was collected, dialyzed against TBS-II, and loaded onto a column of DEAE Sephadex A-25, pre-equilibrated with the same buffer. After washing the column with TBS-II buffer until no protein was found in the washings, the bound protein was eluted with 100 mM choline chloride in the same buffer. The eluted protein was dialyzed extensively against TBS-I to remove the choline chloride, concentrated by lyophilization, and then dialyzed again against the same buffer and stored at 4°C. Alternatively, the protein was lyophilized to a powder and stored at −20°C. Both types of sample were found to bind to phosphatidylcholine membranes, or to DEAE Sephadex A-25, without any noticeable decrease in the activity over several months of storage. Purity of PDC-109 was assessed by SDS-PAGE (Laemmli, 1970) where the protein moved as two closely spaced bands of $M_r \sim 13$ kDa. Gels of 10% or 12% acrylamide were routinely used.

Binding of PDC-109 to DMPC and DMPC/cholesterol mixtures

The binding of PDC-109 to DMPC multilamellar vesicles and to DMPC MLVs containing 25 mol % cholesterol was investigated by turbidimetry. The lipid/lipid mixture was dissolved in CH_2Cl_2, and aliquots corresponding to 1.0 mg of lipid each were transferred to glass test tubes and the solvent was removed in a nitrogen gas stream. After removing the traces of solvent by vacuum desiccation for over 3 h, the lipid film was hydrated with 1.0 ml of 10 mM Hepes, 1 mM EDTA, 0.15 M NaCl, pH 7.4 buffer (HBS), or with different amounts of protein solution in the same buffer. An appropriate volume of buffer was added in the latter case to make up the volume to 1.0 ml. The samples were warmed to ~30°C and were mildly vortexed several times and then subjected to 10 freeze-thaw cycles to get a homogenous suspension. Alternately, samples were prepared by first hydrating the lipid film with HBS, followed by the addition of PDC-109 and 10 freeze-thaw cycles. Turbidity of the samples was measured at 330 nm in a Shimadzu UV-3101PC UV-Vis-NIR double beam spectrometer using 1-cm-path-length cells. Essentially similar results were obtained with both methods of sample preparation.

The PDC-109/DMPC recombinants of high protein/lipid ratio (P/L > 2.0 w/w) were further analyzed by gel filtration on Sepharose CL6B (cf. Moreau and Manjunath, 1999). The gel filtration column (1.7 × 50 cm) was equilibrated with HBS and calibrated with standards with M_r values in the range of 60,000 to 2×10^6 Da. Fractions were monitored by checking absorption at 280 nm for protein and 330 nm for turbidity.

Electron spin resonance spectroscopy

ESR spectra were recorded on a Varian E-12 Century Line 9-GHz ESR spectrometer. Samples were placed in 100-μl glass capillaries and flame sealed. The capillaries were placed in a standard 4-mm quartz sample tube containing light silicone oil for thermal stability. The temperature of the

sample was maintained constant by blowing thermostatted nitrogen gas through a quartz dewar. Spectra were recorded using the following instrumental settings: scan width, 100 G; scan time, 4 min; time constant, 0.25 s; modulation amplitude, 1.25 G; incident power, 10 mW. Values of the outer hyperfine splitting, $2A_{max}$, were determined by measuring the difference between the low-field maximum and the high-field minimum. The error in measuring the position of the maximum and minimum is ~0.1–0.3 G depending on the degree of motional line broadening; therefore the overall error in the estimation of $2A_{max}$ is ~0.2–0.6 G. Spectral subtractions were performed essentially as described by Marsh (1982), using a program written by Dr. Jörg H. Kleinschmidt. Reference spectra for the motionally restricted components were selected from a library of spectra of 14-PCSL bound to the proteolipid protein from bovine myelin, recorded at different temperatures. Reference spectra for the fluid components were chosen from a library of spectra obtained by recording the ESR spectra of 1 mol % 14-PCSL in egg PC at different temperatures. Subtraction end points were established by overlaying the difference spectrum with the matching fluid reference spectrum. To improve precision, the spectra were expanded vertically in the outer wings. Uncertainty in the determination of the end point is in the region of ±0.02–0.05, and this corresponds also to the repeatability of determinations of the subtraction factor, *f*. This estimate does not include the systematic error associated with imperfections in the match with the reference spectra.

Sample preparation

Samples for ESR spectroscopy were prepared as follows. The lipid and 1 mol % of the spin label were co-dissolved in CH_2Cl_2, and a thin film of the lipid was produced by evaporating the solvent with dry nitrogen gas. Final traces of solvent were removed by subjecting the sample to vacuum desiccation for at least 3 h. The sample was then hydrated with 100 μl of HBS buffer and vortexed. The lipid suspension thus obtained was transferred into a 100-μl glass capillary and pelleted in a tabletop centrifuge. Excess supernatant was removed and the capillary was flame-sealed. Samples containing protein-lipid complex were prepared in a similar manner except that the lipid film was hydrated directly with the protein solution in HBS and subjected to at least five freeze-thaw cycles before transferring to the glass capillaries. It was found that the samples containing protein could no longer be pelleted, and therefore the sample was used as such for ESR studies. In view of this limitation, the volume of protein solution added was minimized by using a high concentration protein (>30 mg/ml), such that the amount of sample in the ESR cavity was maximized.

Protein assay

Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. The concentration of purified PDC-109 was estimated from its extinction coefficient at 280 nm of 2.5 for a 1 mg/ml sample concentration (Calvete et al., 1996).

RESULTS

Lipid turbidity and binding of PDC-109 to DMPC membranes

Preliminary experiments on the binding of PDC-109 to multilamellar vesicles of DMPC indicated that binding results in a partial solubilization of the vesicles as could be observed by a decrease of the sample turbidity. Therefore, binding and solubilization were monitored by measuring turbidity of the samples at a wavelength of 330 nm. Fig. 1 gives the results of binding experiments where the turbidity

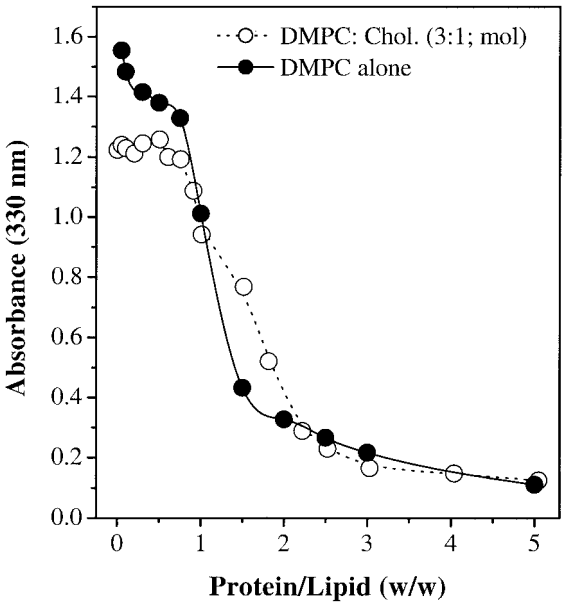

FIGURE 1 Binding of PDC-109 to DMPC multilamellar vesicles and DMPC:cholesterol mixture, monitored by turbidimetry. The samples were prepared by directly hydrating 1.0 mg of the lipid with an appropriate amount of the protein and making up the volume to 1.0 ml. The samples were then subjected to 10 freeze-thaw cycles, and the turbidity was measured spectrophotometrically at 330 nm. Temperature = 30°C.

of MLVs of DMPC as well as DMPC:cholesterol (3:1; mol/mol) was monitored as a function of the protein/lipid (P/L) ratio. From this figure it is seen that the sample turbidity decreases very little at low P/L ratios (up to ~0.75 P/L weight ratio), both for DMPC as well as for DMPC:cholesterol (3:1; mol/mol). However, as the P/L ratio is increased further, the sample turbidity decreases steeply up to a P/L weight ratio of ~1.5 and then decreases more gradually as the P/L ratio is increased further. The results obtained were qualitatively very similar irrespective of whether the lipid film was directly hydrated with the protein solution or protein solution was added to the prehydrated lipid dispersion. Very similar results were obtained with DMPC membranes containing 25 mol % cholesterol, except that the decrease in absorbance was less steep and achieved a limiting low value at rather higher protein content (~2:1 w/w) (see Fig. 1). In view of these observations, a 2:1 P/L weight ratio was chosen for the ESR spectral studies. Furthermore, to investigate the effect of protein binding on the lipid chain-melting phase transition, membranes made up of DMPC alone were used, as this lipid gives a well-defined gel-fluid phase transition.

Effect of binding of PDC-109 on lipid phase transition and acyl chain dynamics

ESR spectra of phosphatidylcholine spin-labeled at the C-5 position of the *sn*-2 chain (5-PCSL) in DMPC membranes,

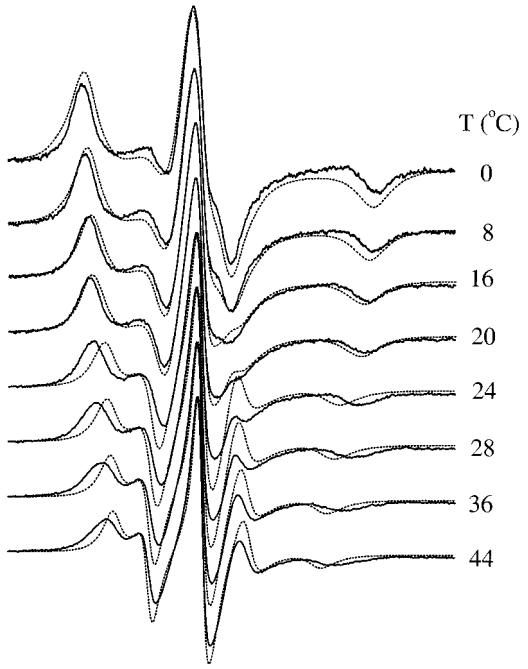

FIGURE 2 ESR spectra of the phosphatidylcholine spin label, 5-PCSL, bearing the nitroxide moiety at the C-5 position of the *sn*-2 acyl chain, in DMPC membranes in the presence and absence of PDC-109 (lipid:protein, 1:2 w/w). Solid lines correspond to spectra recorded from DMPC/PDC-109 recombinants, and dashed lines correspond to the spectra recorded from DMPC membranes alone. The temperature at which the spectra were recorded is indicated in the figure. The spectral width is 100 G.

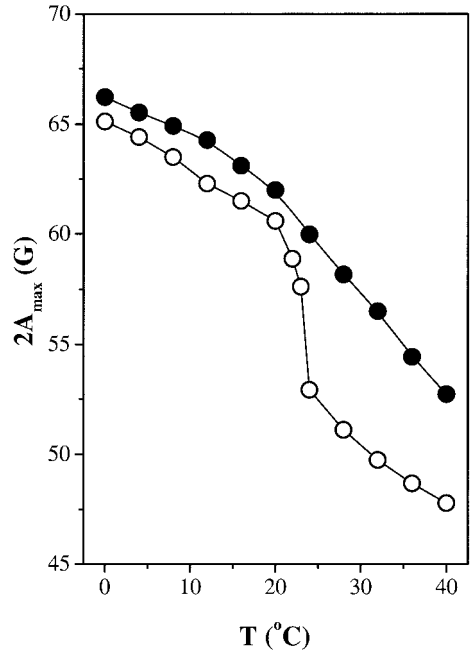

FIGURE 3 Temperature dependence of the outer hyperfine splitting, $2A_{max}$, for the 5-PCSL spin label in DMPC membranes (○) and in DMPC/PDC-109 recombinants (●).

in the absence and in the presence of 2:1 (w/w) ratio of added PDC-109, are shown in Fig. 2. The spectra recorded in the absence of protein (dashed lines) exhibit an abrupt change in the line shapes and outer hyperfine splittings ($2A_{max}$) at ~24°C, resulting from the increase in lipid chain mobility at the chain-melting phase transition. Those recorded in the presence of PDC-109 (solid lines) do not show any such abrupt changes; the changes observed in the ESR spectra of the PDC-109-bound sample with increasing temperature are more gradual. ESR spectra of 5-PCSL in DMPC membranes at 28°C show a progressive increase in outer hyperfine splitting with increasing PDC-109/DMPC ratios up to ~2:1 (w/w) and then remain essentially constant (data not shown). This behavior is consistent with the binding stoichiometry extrapolated from the steep change in turbidity that is shown in Fig. 1.

Temperature dependences of the outer hyperfine splitting, $2A_{max}$, for 5-PCSL in DMPC membranes, in the presence and in the absence of a 2:1 (w/w) ratio of PDC-109, are given in Fig. 3. It is clearly seen from this figure that the chain-melting phase transition, which is indicated for DMPC membranes alone by an abrupt decrease in the value of $2A_{max}$ at ~23°C, is strongly perturbed by the binding of PDC-109. At any given temperature, the outer hyperfine splitting is consistently higher in the presence than in the absence of PDC-109. This indicates that the protein interacts with DMPC membranes in both the gel and fluid phases.

Positional dependence of lipid chain perturbation by PDC-109

To investigate whether the binding of PDC-109 to DMPC membranes is accompanied by the interaction of the protein with the hydrophobic interior of the lipid membrane, that is, to investigate whether the protein or a part of it inserts into the interior of the lipid bilayer, ESR studies were performed with spin-labeled phosphatidylcholines, *n*-PCSL, bearing the nitroxide spin label at different positions down the *sn*-2 acyl chain of the lipid. The ESR spectra of different positional isomers of phosphatidylcholine, in DMPC membranes and in samples containing 2:1 (w/w) ratio of added PDC-109, recorded at 0°C in the gel phase region of hydrated DMPC, are given in Fig. 4. From these spectra it is evident that in the gel phase, protein binding decreases the mobility of the lipid chains of DMPC at all positions up to the 14th C atom.

ESR spectra of *n*-PCSL in dispersions of DMPC alone and in samples containing PDC-109, recorded at 28°C, corresponding to the liquid-crystalline phase of the host lipid, are given in Fig. 5. Whereas the ESR spectra of the spin-labels at positions close to the headgroup ($n = 5$ and 8) are broadened by protein binding, possibly indicating the presence of overlapping components, those that are closer to

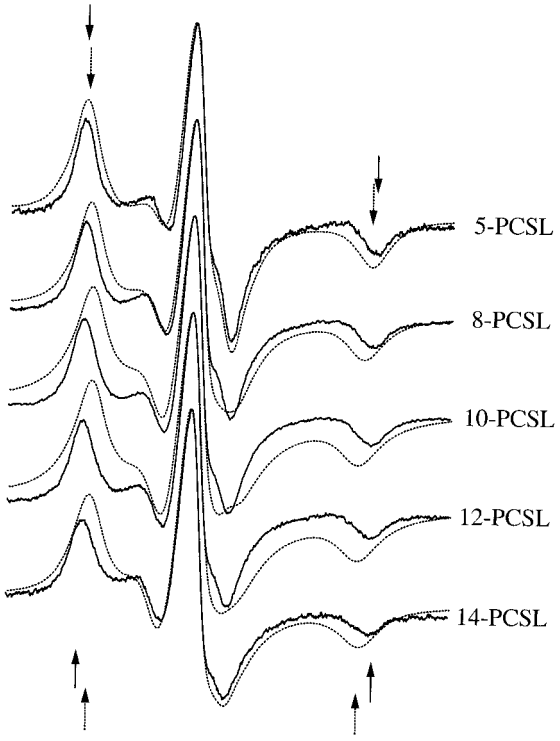

FIGURE 4 ESR spectra of phosphatidylcholine spin labels, *n*-PCSL, at 0°C in the gel phase of DMPC membranes, in the presence and absence of PDC-109 (lipid:protein, 1:2 w/w). Dotted lines correspond to spectra recorded from DMPC membranes alone, and solid lines correspond to the spectra recorded from DMPC membranes in the presence of PDC-109. Arrows indicate the outer hyperfine splitting, $2A_{max}$. The spectral width is 100 G.

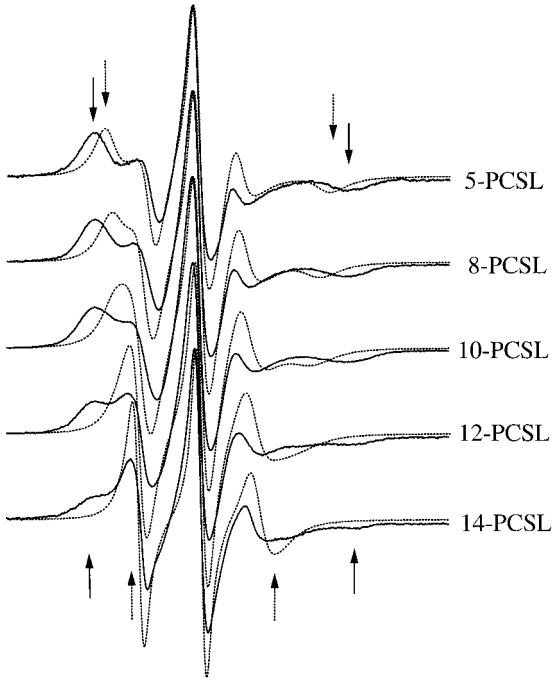

FIGURE 5 ESR spectra of phosphatidylcholine spin labels, *n*-PCSL, at 28°C in the fluid phase of DMPC membranes, in the presence and absence of PDC-109 (lipid:protein, 1:2 w/w). Dotted lines correspond to spectra recorded from DMPC membranes alone, and solid lines correspond to the spectra recorded from DMPC membranes in the presence of PDC-109. Arrows indicate the outer hyperfine splitting, $2A_{max}$. The spectral width is 100 G.

the methyl end of the chain ($n = 12$ and 14) clearly consist of two components. Notably, the spectrum of 14-PCSL closely resembles the spectra obtained with integral transmembrane proteins that have been reconstituted into lipid membranes (see, e.g., Marsh and Horváth, 1998). One component in this spectrum is similar to the fluid lipid spectrum (shown by the dashed line). The second component (resolved in the outer wings of the spectrum) has a much larger outer hyperfine splitting, $2A_{max}$, and represents a lipid population whose acyl chains are in direct contact with the protein.

A plot of the values of $2A_{max}$ as a function of chain-labeling position, obtained for the phosphatidylcholine spin labels, *n*-PCSL, in membranes of DMPC alone and in DMPC/PDC-109 recombinants (corresponding to the motionally restricted component, where the lines could be resolved) at 28°C, is given in Fig. 6. Whereas the values of $2A_{max}$ obtained for the lipid membranes alone decrease as the spin-label position is moved down the *sn*-2 chain of the lipid, thus displaying the chain flexibility gradient characteristic of fluid lipid membranes, the values of $2A_{max}$ for the motionally restricted component remain approximately constant, and are consistently larger, throughout the chain. These observations further substantiate the interpretation given above that PDC-109 penetrates into the hydrophobic interior of the lipid membrane and interacts directly with the acyl chains.

Interaction of PDC-109 with different spin-labeled lipid species

To investigate the selectivity of PDC-109 for different lipids, DMPC vesicles containing 1 mol % of different phospholipid spin label species bearing the nitroxide moiety in the *sn*-2 acyl chain at the 14th C atom were prepared. In addition, samples were also prepared with probe amounts of two steroid-based spin labels, the cholestane spin label and the androstanol spin label. ESR spectra of the membrane samples in the presence and in the absence of the protein were recorded in the fluid-phase region of the lipid. A comparison of the spectra obtained in the presence of PDC-109 and in its absence, recorded at 28°C, is given in Fig. 7. From this figure it is seen that, as for 14-PCSL, all the spectra obtained in the presence of PDC-109 are composed of two components. There is a fluid-like component, which is similar to that of the spin label in lipid membranes alone, and another component that corresponds to a more motionally restricted spin-label population. The relative amounts

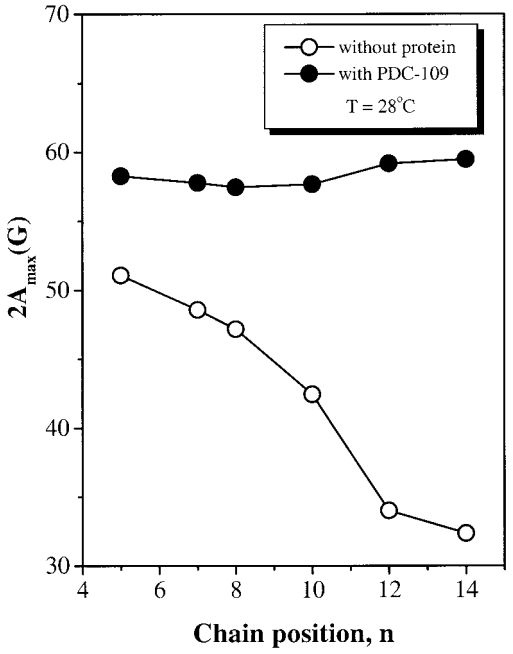

FIGURE 6 Positional dependence of the outer hyperfine splitting, $2A_{max}$, for phosphatidylcholine spin labels, *n*-PCSL, in DMPC membranes (○) and in DMPC/PDC-109 (lipid:protein, 1:2 w/w) recombinants (●), at 28°C in the fluid phase. The data given for $n = 12$ and 14 in DMPC/PDC-109 recombinants correspond to the motionally restricted component of the two-component spectra.

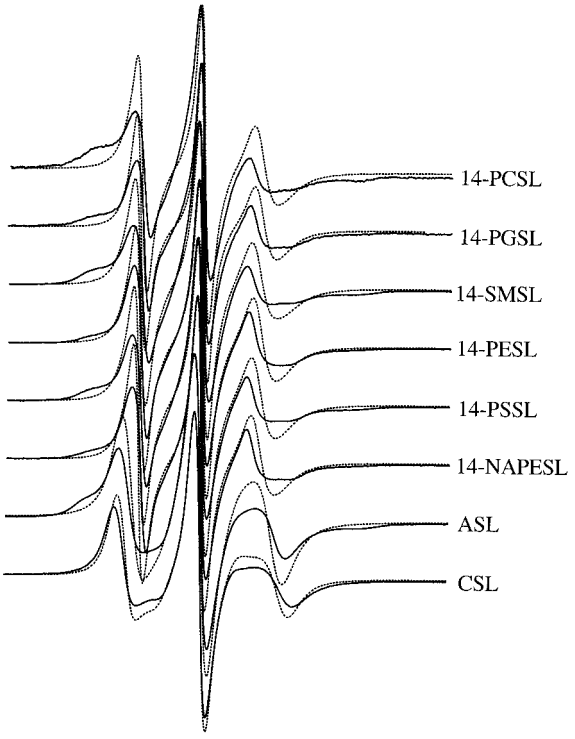

FIGURE 7 ESR spectra of different phospholipid spin labels, 14-XXSL, bearing the spin label on the 14th C atom of the *sn*-2 chain, as well as the cholestane spin label (CSL) and androstanol spin label (ASL), in the fluid phase of DMPC membranes (· · ·) and of DMPC/PDC-109 (lipid:protein, 1:2 w/w) recombinants (——). Phospholipid spin labels are: 14-PCSL (phosphatidylcholine), 14-PGSL (phosphatidylglycerol), 14-SMSL (sphingomyelin), 14-PESL (phosphatidylethanolamine), 14-PSSL (phosphatidylserine), and 14-NAPESL (*N*-acyl phosphatidylethanolamine). Spectra were recorded at 28°C. The spectral width is 100 G.

of these two components differ between the different lipid species, reflecting the selectivity of interaction with the PDC-109 protein.

The two components in the ESR spectra of different spin labels obtained in the presence of PDC-109 can be resolved by spectral subtraction and the contribution of each component to the overall spectrum quantified. This is shown in Fig. 8 for several of the spin labels in a DMPC host matrix, in the presence of a 2:1 weight ratio of added PDC-109. The spin label in each set of spectra is indicated on the right. In each set of four spectra the top pair correspond to the composite spectrum from the lipid-protein complex (solid line) and an ESR spectrum matching the motionally restricted component alone (dotted line). The lower pair of spectra in each set of spectra corresponds to the fluid component obtained from subtraction (solid line) and a matching fluid spectrum (dotted line). The relative amounts of the motionally restricted components (fraction, *f*) for the different spin-labeled lipids in a DMPC host matrix in the presence of PDC-109 are given in Table 1. These values correspond to the fraction of the total double-integrated intensity that must be subtracted from the composite first-derivative spectrum of the PDC-109/lipid complex to obtain the fluid-component end point. Note that the spectra in Fig. 8 are all normalized to the same maximum line height, and therefore the difference spectra do not reflect their true relative intensities.

DISCUSSION

Considering the fact that PDC-109 induces efflux of choline phospholipids and cholesterol from spermatozoa by binding to them upon ejaculation, the molecular mechanism of this process is of great interest. Especially, because this lipid efflux from spermatozoa promotes capacitation, a clear understanding of the efflux of phosphatidylcholine and cholesterol mediated by PDC-109 could potentially lead to the development of new anti-fertility drugs. In view of this, in the present study we have characterized the interaction of PDC-109 with phosphatidylcholine membranes by using turbidity measurements and spin-label ESR spectroscopy. Further, the lipid selectivity of this protein has been investigated in some detail by using spin-labeled analogs of different phospholipids and steroid derivatives.

Solubilization of DMPC membranes by PDC-109

As seen from Fig. 1, binding of PDC-109 to DMPC MLVs resulted in a decrease in the sample turbidity, indicating that the size of the lipid assemblies is decreased upon protein

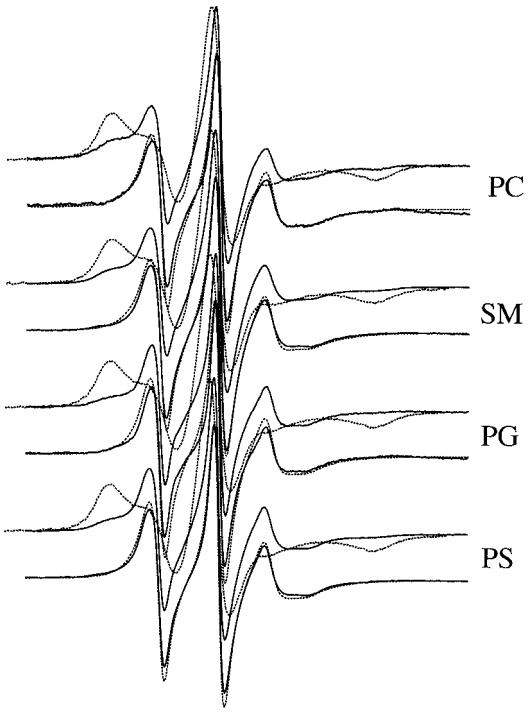

FIGURE 8 Representative spectral subtractions for 14-position spin labels. Spectra of 14-PCSL, 14-SMSL, 14-PGSL, and 14-PSSL in DMPC/PDC-109 complexes are shown. In each set of four spectra the upper pair correspond to the composite spectrum of the PDC-109/lipid complex (——) and the motionally restricted component spectrum (· · ·) that is used for subtraction from the composite spectrum. The lower pair of spectra correspond to the subtraction result (——) and a matching fluid lipid reference spectrum (· · ·). The spin label used for obtaining the spectrum of the PDC-109 lipid complex in each case is indicated in the figure. Spectra are all (including difference spectra) displayed normalized to the same maximum line height.

TABLE 1 Fraction (*f*) of motionally restricted spin-labeled lipid and relative association constants, K_r, normalized to that for phosphatidylcholine (K_r^{PC}) obtained from spectral subtractions with the ESR spectra of different spin labels in DMPC/PDC-109 complexes

Spin label	Temperature (°C)	Motionally restricted fraction (*f*)	K_r/K_r^{PC}
14-PASL (pH 8.5)	28	0.55	1.38
14-PCSL	28	0.47	1.0
14-SMSL	28	0.47	1.0
14-PASL (pH 6.0)	28	0.46	0.96
14-PGSL	28	0.35	0.61
14-PSSL	28	0.35	0.61
14-NAPESL	28	0.21	0.30
14-PESL	28	0.23	0.34
ASL	32	0.35	0.61

The P/L ratio for all the samples was 2 (w/w). The *f* values are subject to an error of $\sim\pm0.02$–0.05 due to uncertainties in the spectral subtractions. For CSL, the motionally restricted spectral component was too small to quantify reliably.

binding. Gel filtration on Sepharose CL6B gave an apparent mass of $\sim1.3 \times 10^6$ Da for the particles obtained upon binding of PDC-109 to DMPC MLVs. Recent experiments by Manjunath and co-workers (Thérien et al., 1997, 1998) indicate that PDC-109 and other BSP proteins promote sperm capacitation by removal of cholesterol and choline phospholipids from sperm plasma membrane. The lipid efflux particles resulting from binding of PDC-109 to human fibroblasts (a cell model used to study the lipid efflux) were shown to be made up of BSP proteins, cholesterol, and choline-containing phospholipids, with a size of ~80 nm (Moreau and Manjunath, 1999). Our observations indicate that most probably the binding of PDC-109 to DMPC MLVs also leads to the formation of such particles. Efflux of choline phospholipids and cholesterol would then be linked to the ability of BSP proteins to partially solubilize PC-containing lipid membranes.

The results presented in Fig. 1 show that, in addition to MLVs of DMPC alone, DMPC MLVs containing 25 mol % cholesterol are also solubilized by PDC-109 at high P/L ratios. The threshold required for complete solubilization, however, is higher. Calvete and co-workers (Gasset et al., 2000) have shown that the leakage of internal contents from DOPC unilamellar vesicles that is induced by PDC-109 becomes considerably reduced in the presence of cholesterol. Whereas there are certain parallels between these two sets of results, they refer to very different types of experiments. The work here concerns the fragmentation of very large MLVs into smaller particles. That of Gasset et al. (2000) involves the permeability/leakage of small, 90-nm-diameter, unilamellar vesicles.

Stoichiometry of PDC-109/DMPC interaction

Fig. 1 represents the titration of DMPC dispersions with PDC-109. Because DMPC was hydrated with the protein-containing solution, all lipid is available for interaction with PDC-109. Extrapolating the steeply changing part of the turbidity to the quasi-saturation level yields a protein/lipid stoichiometry of ~1.7 w/w, i.e., a mole ratio of ~11 DMPCs per PDC-109. For comparison, fluorescence titration of PDC-109 with small unilamellar DMPC vesicles yielded a stoichiometry of ~12 DMPCs/PDC-109 (Gasset et al., 2000). A corresponding fluorescence titration with PC, also in 2:1 mixtures with phosphatidylethanolamine or phosphatidylserine, yielded a stoichiometry of ~10 PCs/PDC-109 (Müller et al., 1998). Note that in both these latter cases, the PDC-109/PC ratios used in the titration were beyond that at which a drastic decrease of particle size is indicated in Fig. 1. The ESR spectra of PC with a short (five C atoms) *sn*-2 chain bearing the nitroxide on the 4th C atom were found to contain a motionally restricted component with a stoichiometry of ~11 PCs/PDC-109 (Müller et al., 1998). Evidently, in this study, the spin labels of all the PCs with short *sn*-2 chain with which PDC-109 interacts contact the protein more or less directly.

Simple volumetric estimates show that a compact globular protein of molecular weight 13 kDa and partial specific volume ~0.7 cm^3/g has a cross-sectional area in the range of ~6–7.5 nm^2. A protein of this size would therefore interact with ~10–12 DMPC molecules (cross-sectional area ~0.6 nm^2) at the membrane surface. These estimates therefore suggest that the PDC-109/PC binding stoichiometries found experimentally correspond approximately to complete coverage of the membrane surface by the protein.

Membrane penetration by PDC-109

The spectra given in Figs. 4 and 5 show that binding of PDC-109 to DMPC membranes affects the mobility of the acyl chains up to the 14th C atom in the gel phase as well as in the fluid phase, although the changes observed in the fluid phase are certainly more marked. In particular, the spectra of the spin labels bearing the nitroxide moiety at positions close to the chain methyl terminus (12-PCSL and 14-PCSL) show two components in the fluid phase, indicating a direct interaction between the protein and the spin-labeled chain segment. Also, the outer peaks in the spectra of 5-PCSL at 36°C and 44°C in Fig. 2 are asymmetric. This suggests that the spectra are two-component even up to this position of chain labeling. The spectral resolution is not so good for 5-PCSL, however, because of the larger outer hyperfine splitting of the fluid component, relative to that for 12-PCSL or 14-PCSL. These results all indicate that the protein, or a part of it, inserts into the membrane interior upon binding and comes into direct contact with the lipid chains. The reduction in cooperativity of the lipid packing that is associated with abolition of the chain-melting phase transition (see Fig. 3) is also consistent with this conclusion.

The extent of motional restriction of the lipid chains by PDC-109 is also a significant indicator of the nature of the protein-lipid interaction. The value of the outer hyperfine splitting for 14-PCSL at 28°C is relatively large: $2A_{max}$ = 59.5 G (see Fig. 6). This can be compared with corresponding values obtained previously for 14-position labels interacting with membrane-penetrant sections of different peripheral and integral proteins (Marsh and Horváth, 1998). The value of $2A_{max}$ for PDC-109 is considerably greater than those for the peripheral membrane proteins apocytochrome *c* ($2A_{max}$ = 49.5 G) and myelin basic protein ($2A_{max}$ = 53.0 G). It is closer to those for the integral proteins, myelin proteolipid protein ($2A_{max}$ = 62.8 G) and cytochrome *c* oxidase ($2A_{max}$ = 60.9 G; Knowles et al., 1979). A rather intimate association of the lipid chains with PDC-109 is therefore implied. This is rather unusual for a soluble protein that recognizes the lipid headgroup in a specific manner (cf. Swamy and Marsh, 1997), and PDC-109 could be unique in this respect. Because PDC-109 undergoes a conformational change upon binding to DMPC membranes (Gasset et al., 1997), it appears likely that the initial step in the membrane binding by PDC-109 is the recognition of the phosphorylcholine headgroup of choline phospholipids by the protein, resulting in a conformational change, ultimately leading to the penetration of the protein segments into the hydrophobic interior of the membrane. The biexponential kinetics of the binding reaction as observed by Müller et al. (1998) is consistent with this model.

At saturation binding of PDC-109 (i.e., 2:1 w/w), approximately half of the spin-labeled phosphatidylcholines are motionally restricted directly by PDC-109 (see Table 1). This corresponds to a rather low lipid/protein stoichiometry of ~5–6 mol/mol. Evidently, only part of the PDC-109 protein penetrates the membrane. Approximately 26 lipids could be accommodated around the perimeter of the protein in a compact globular form, if it is transmembrane (see Marsh 1997), but half this number if it penetrates the membrane only partially. Additionally, it is possible that the protein is partially aggregated at saturation binding, which would further reduce the intramembrane perimeter accessible to lipid. The stoichiometry of ~11 motionally restricted lipids found by Müller et al. (1998) at high lipid/protein ratio that was mentioned already is possibly relevant in this connection.

Relation to protein structure

In this section we attempt to identify those parts of the protein sequence that are most likely to penetrate the membrane. This is done by standard hydropathy analysis, but using two separate scales corresponding to the interfacial region and hydrophobic core of the membrane, respectively (see White and Wimley, 1999).

Fig. 9 gives the free energy, averaged over a sliding window of seven residues, for transfer of PDC-109 to the interfacial region of the membrane (solid line). Individual-residue transfer free energies are those determined by White and Wimley (1999). Regions of this length whose transfer is energetically favorable are indicated by the horizontal solid lines in Fig. 9. These regions are contained within the two FnII domains and constitute a considerable part of the protein. The corresponding hydropathy profile for transfer from the interfacial region of the membrane to the hydrophobic core is indicated by the dashed lines. Even for relatively short seven-residue regions, a substantially smaller portion of the protein is predicted to penetrate the hydrophobic regions of the membrane than partitions to the membrane interface. This is in agreement with the relatively low stoichiometry of motionally restricted lipids that is detected by spin-label ESR.

When the size of the window is increased to nine residues or more, no sections of the protein with this length are predicted to have transfer free energies that favor a location in the hydrophobic core of the membrane. A similar situation was found for the myelin basic protein (Sankaram et al., 1989). No very extended hydrophobic stretches are present

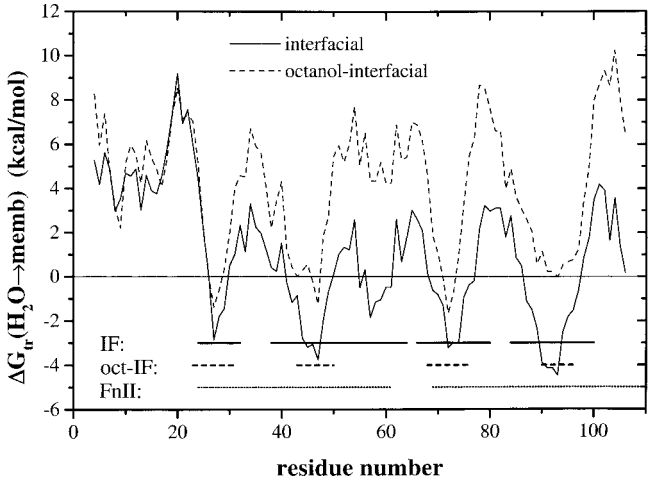

FIGURE 9 Hydropathy profile for PDC-109 (Bräuer and Scheit, 1991), calculated with the interfacial (——) and octanol minus interfacial (— — —) hydrophobicity scales of White and Wimley (1999). Plotted is the free energy of transfer from water to the membrane interface (——) and from the interface to the hydrophobic core of the membrane (— — —), by using a seven-residue window. Solid and dashed horizontal lines represent energetically favorable regions for transfer to the membrane interface (IF) and to the hydrophobic core (oct-IF), respectively. Dotted horizontal lines designate the two FnII domains.

in the sequence of this latter protein, although a motionally restricted lipid population was detected on binding to negatively charged lipids. As might be expected from the above, no transmembrane sections are predicted for PDC-109 with a window length of 19 residues. However, energetically favorable transfer is still predicted to the membrane interface: for the sections of the protein consisting of residues 43–76 and 82–100. This relatively strong propensity of the protein to partition to the membrane interface will certainly potentiate penetration of the hydrophobic core by shorter sections of the protein within these regions (see Fig. 9).

Of course, FnII domains could not be situated in the interfacial region of the membrane in their entirety if PDC-109 maintains its solution conformation on binding to lipid. However, solution NMR studies of the second FnII domain of PDC-109 and the homologous first FnII domain of fibronectin (Constantine et al., 1992; Pickford et al., 1997) have shown that five of the highly conserved clusters of aromatic amino acid residues in these domains form a solvent-exposed hydrophobic surface. It is therefore likely that this surface, which constitutes the binding site for collagen, is also that which binds to lipid membranes. Penetration of these hydrophobic side chains into the membrane may also, at least in part, be responsible for the direct immobilization of the lipid chains that we observe by ESR spectroscopy.

To summarize, candidate regions of the sequence for which penetration of the hydrophobic core of the membrane is energetically favorable are restricted to the FnII domains, because the N-terminal region outside these domains bears the acidic residues. Candidate penetrant sequences are short, seven residues or less, and are indicated by the horizontal dashed bars in Fig. 9. The net transfer free energies are favorable but relatively small and therefore are probably insufficient to ensure tight binding of the protein. The latter is presumably determined by interfacial interactions and the specific interaction with the headgroups of choline-containing phospholipids. The situation is somewhat analogous to that of the myelin basic protein (Sankaram et al., 1989) and apocytochrome *c* (Görrissen et al., 1986; Marsh, 2001). Both these proteins partially penetrate negatively charged lipid membranes but are displaced from the membrane at high ionic strength.

Lipid selectivity

A clear pattern of selectivity in interaction of PCD-109 with different lipid species is evident from Table 1. The lipid affinity can be expressed thermodynamically in terms of the association constants, K_r, for interaction of the different spin-labeled lipids with PDC-109, relative to the background DMPC host lipid. The experimentally determined fractions, f, of motionally restricted lipid depend directly on these relative association constants. Normalized relative to the value, K_r^{PC}, for spin-labeled phosphatidylcholine, the relative association constants are given by (Marsh, 1985):

$$K_r/K_r^{PC} = (1/f_{PC} - 1)/(1/f - 1), \qquad (1)$$

where f_{PC} (0.47) is the fraction of motionally restricted 14-PCSL. These values of K_r/K_r^{PC} are also listed in Table 1. Equation 1 is derived from the equation for equilibrium lipid-protein association, taking into account the fact that the lipid/protein ratio is the same for all samples.

Based on the data given in Fig. 7 and Table 1, the lipid selectivity of PDC-109 is in the following order: phosphatidic acid (pH 8.5) > phosphatidylcholine ≈ sphingomyelin ≥ phosphatidic acid (pH 6.0) > phosphatidylglycerol ≈ phosphatidylserine ≈ androstanol > phosphatidylethanolamine ≥ *N*-acyl phosphatidylethanolamine ≫ cholestane. Though the highest selectivity is seen for phosphatidic acid dianion, the physiologically irrelevant pH of 8.5 where it is seen coupled with the fact that phosphatidic acid is normally present only at very low concentrations in normal membranes makes this observation less important. The selectivity observed for the phosphocholine-containing lipids, phosphatidylcholine and sphingomyelin, is the highest among other lipids for their interaction with PDC-109. This is a highly unusual situation compared with spin-label results obtained on the selectivity of lipid interactions with integral membrane proteins (Marsh and Horváth, 1998). In the latter case, although the selectivity of interaction is not solely electrostatic in origin, the highest selectivities are obtained for anionic lipids. Neither phosphatidylcholine nor sphin-

gomyelin display a preferential interaction with any of the integral proteins studied. Also, peripheral membrane proteins do not interact preferentially with phosphatidylcholine (Sankaram and Marsh, 1993).

The selectivity pattern that emerges from Table 1 is consistent with the findings of Desnoyers and Manjunath (1992), who investigated the selectivity with a more limited range of lipid species. Their studies involved the binding to lipids coated on plastic plates, as opposed to the lipid probes in bilayer membranes used in the present study. Also, the selectivity for phosphatidylcholine over phosphatidylserine and phosphatidylethanolamine found here is consistent with the attenuation of binding to PC vesicles containing admixtures of these latter two lipids that was observed by Müller et al. (1998).

The results obtained with the steroid probes, ASL and CSL, deserve comment in view of the involvement of BSP proteins in cholesterol efflux (Thérien et al., 1998, 1999). We find that PDC-109 exhibits considerable selectivity for the ASL. This is a sterol analog that contains the 17β-OH group attached to the steroid nucleus. On the other hand, the CSL in which the 3β-OH group of the cholesterol structure is replaced by the nitroxide doxyl moiety is very poorly recognized by the protein. This suggests that the hydroxy group of cholesterol is important for the recognition by the protein, whereas the alkyl tail at the 17-position is not particularly relevant for this interaction. In this connection, it should be noted that spin-labeled androstanol displays an affinity for the nicotinic acetylcholine receptor like that of cholesterol (Ellena et al., 1983) and interacts with the polyene antibiotic amphotericin in a manner similar to that of cholesterol (Aracava et al., 1981).

CONCLUSIONS

The ESR results presented in this study demonstrate that PDC-109 binds to phospholipid membranes by specific interaction with choline phospholipids. The binding results in an immobilization of the lipid acyl chains and also solubilizes multilamellar liposomes made up of PC and of PC/cholesterol. Although PDC-109 exhibits the highest selectivity for the choline phospholipids (PC and SM), it also shows considerable selectivity for other phospholipids such as phosphatidylglycerol and phosphatidylserine. The distinctly higher selectivity observed for the ASL as opposed to the CSL shows that the hydroxyl group of the sterol plays a crucial role in the interaction of the steroid with PDC-109 and hence could be important in the process of cholesterol efflux. The specific recognition of PC and SM by PDC-109 is a significant result because the zwitterionic lipids PC, phosphatidylethanolamine, and SM have long been considered to play a passive structural role in biological membranes. The present results demonstrate that the phosphorylcholine moiety of PC (or the sphingolipid analog SM) can be specifically recognized by proteins, and such recognition can mediate biologically important processes such as cholesterol efflux from the sperm cell membranes. It would be interesting to see whether there are other choline-binding proteins in nature that recognize PC/SM in their interaction with biological membranes.

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