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Effect of cholesterol on the interaction of seminal plasma protein, PDC-109 with phosphatidylcholine membranes

Musti J. Swamy[a,*], D. Marsh[b], V. Anbazhagan[a], M. Ramakrishnan[a]

[a] *School of Chemistry, University of Hyderabad, Hyderabad 500 046, India*
[b] *Max-Planck Institut für biophysikalische Chemie, 37070 Göttingen, Germany*

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Abstract Binding of PDC-109, the major protein of the bovine seminal plasma, to sperm plasma membrane results in an efflux of cholesterol and choline phospholipids, a necessary event before capacitation can occur. The selectivity of PDC-109 for different spin-labelled phospholipids and sterol probes in dimyristoylphosphatidylcholine (DMPC) host matrix has been characterized earlier by EPR spectroscopy [Ramakrishnan, M., Anbazhagan, V., Pratap, T.V., Marsh, D. and Swamy, M.J. (2001) Biophys. J. 81, 2215–2225]. In this report the effect of cholesterol on the interaction of PDC-109 with DMPC membranes has been investigated by spin-label EPR spectroscopy. The results indicate that the presence of cholesterol leads to an increased association of different phospholipid as well as sterol probes, thus modulating the interaction of PDC-109 with phospholipid membranes. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: BSP-A1; Lipid–protein interaction; Lipid bilayer; Cholesterol efflux; Phosphatidylcholine; Sphingomyelin

1. Introduction

In mammals, the seminal plasma carries the ejaculated spermatozoa through the female genital tract to their final destination, the uterus. It is a highly complex fluid containing a variety of small molecules, but interestingly contains only proteins as the high molecular weight constituents, whereas other biopolymers such as polysaccharides and nucleic acids are not present [2]. Among the different mammalian species, considerable attention has been focused on the proteins from bovine seminal plasma. There are four closely related proteins in the bovine seminal plasma, namely BSP-A1, BSP-A2, BSP-A3 and BSP-30kDa protein, which bind to sperm plasma membrane [3–5]. Collectively these proteins are referred to as *bovine seminal plasma proteins*, or in short, as *BSP proteins*. BSP-A1 and BSP-A2 are identical in their primary structure of 109 amino acid residues and differ only in glycosylation. A mixture of the glycosylated and unglycosylated forms of this protein is referred to as PDC-109. The 109 amino acid residues of this protein are arranged in three parts; a 23-residue amino terminal peptide followed by two tandemly repeating fibronectin type-II (Fn-II)[1] domains [6–8]. PDC-109 is the major protein of the bovine seminal plasma and is present at ~15–25 mg/ml concentration [9]. The three-dimensional structure of the PDC-109/phosphorylcholine complex indicates that the two binding sites for the ligand are on the same face of the protein molecule [10].

BSP proteins bind to sperm cell membranes by their specific interaction with the choline containing phospholipids, namely phosphatidylcholine (PC) and sphingomyelin (SM) [5]. This binding results in an efflux of choline phospholipids and cholesterol, referred to collectively as *cholesterol efflux*. Cholesterol efflux results in a decrease of the cholesterol/phospholipid ratio and promotes capacitation, which is a necessary event before the sperm can undergo acrosome reaction and finally fertilize the egg [11,12].

Each Fn-II domain of PDC-109 binds to one choline phospholipid molecule and both binding sites are necessary for inducing cholesterol efflux [13]. In order to understand at the molecular level how cholesterol efflux is induced by this protein, it is important to investigate its interaction with phospholipid membranes with and without cholesterol. Such studies are expected not only to shed light on the molecular events involved in the capacitation process but can potentially lead to the development of novel anti-fertility drugs. In an earlier study we investigated the interaction of PDC-109 with PC membranes bearing probe amounts of different spin-labelled phospholipids and sterols by electron paramagnetic resonance (EPR) spectroscopy. These studies showed that, in addition to spin-labelled PC and SM, PDC-109 also recognizes phosphatidylserine and phosphatidylglycerol spin labels as well as a cholesterol analogue, androstanol spin label with considerable affinity, whereas very weak interaction was observed with spin-labelled phosphatidylethanolamine [1]. In the present study, the interaction of PDC-109 with model membranes made up of dimyristoylphosphatidylcholine (DMPC) and cholesterol are investigated by spin-label EPR spectroscopy. The results obtained indicate that the interaction of PDC-109 with different phospholipids is increased considerably by the presence of cholesterol.

*Corresponding author. Fax: (91)-40-301 2460 or 0145.
E-mail address: mjssc@uohyd.ernet.in (M.J. Swamy).

Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; SM, sphingomyelin; EPR, electron paramagnetic resonance; 14-PCSL, -PESL, -PSSL, 1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)]stearoyl-*sn*-glycero-3-phosphocholine, -phosphoethanolamine, -phosphoserine; 14-SMSL, *N*-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-sphingosine-1-phosphocholine; CSL, 4′,4′-dimethylspiro(5α-cholestane-3,2′-oxazolidin)-3′-yloxyl; ASL, 17β-hydroxy-4′,4′-dimethylspiro(5α-androstane-3,2′-oxazolidin)-3′-yloxyl; Fn-II, fibronectin type-II domain; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)

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2. Materials and methods

2.1. Materials

Phosphorylcholine chloride (Ca^{2+} salt), choline chloride and Tris base were from Sigma (St. Louis, MO, USA). Sephadex G-50 (superfine), DEAE Sephadex A-25 and DEAE Sephadex A-50 were obtained from Pharmacia Biotech (Uppsala, Sweden). DMPC and cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Stearic acid spin labelled on the 14th C-atom and PC, spin labelled on the 14th C-atom of the *sn*-2 acyl chain (14-PCSL; 1-acyl-2-[*n*-(4,4-dimethyloxazolidine-*N*-oxyl)]stearoyl-*sn*-glycero-3-phosphocholine) were synthesized according to procedures outlined in [14]. Spin-labelled phospholipids with different polar headgroups (14-PESL, phosphatidylethanolamine and 14-PSSL, phosphatidylserine) were prepared from 14-PCSL by phospholipase D-catalyzed headgroup exchange, as described in [14]. Spin-labelled SM (14-SMSL) was prepared as described in [15]. Cholestan spin label (4′,4′-dimethylspiro[5α-cholestane-3,2′-oxazolidin]-3′-yloxyl) and androstanol spin label (17β-hydroxy-4′,4′-dimethylspiro[5α-androstan-3,2′-oxazolidin]-3′-yloxyl) were obtained from Syva (Palo Alto, CA, USA). PDC-109 was purified and characterized as described previously [1]. 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 [16].

2.2. Electron paramagnetic resonance spectroscopy

EPR spectra were recorded on a Varian E-12 Century Line 9 GHz ESR spectrometer. Details of sample preparation and instrumental settings for ESR spectroscopy have been described earlier [1]. Spectral subtractions were performed essentially as described in [17], using a program written by Dr. Jörg H. Kleinschmidt. Details of measuring the values of the outer hyperfine splitting, $2A_{max}$, and spectral subtractions are given in [1]. 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 taken either from spectra of the same spin-labelled lipid in the lipid mixture alone recorded at a slightly lower (by ca. 2–4 degrees) temperature or from a library of spectra of 14-PCSL in lipid extracts from the (Na^+,K^+)-ATPase membranes of *Squalus acanthias* [18]. Reference spectra for the fluid components obtained from DMPC membranes in the presence of PDC-109 were taken from a library of spectra of 14-PCSL in egg yolk PC, recorded at different temperatures [1].

3. Results and discussion

The effect of cholesterol on the stoichiometry of PC association with PDC-109 was studied using a PC probe spin labelled on the 14th C-atom of the *sn*-2 chain. Lipids interacting directly with the protein appear as a second, motionally restricted component in the spin-label EPR spectrum. The effect of cholesterol on the selectivity of lipid association with PDC-109 was studied similarly by using different spin-labelled phospholipid and sterol species.

3.1. Binding of PDC-109 to DMPC/cholesterol membranes

In order to investigate the effect of cholesterol on the binding of PDC-109 to PC membranes, the initial experiments were carried out with fluid DMPC membranes containing different amounts of cholesterol (0–20 wt%) and 1 mol% of spin-labelled PC, 14-PCSL. EPR spectra were obtained in the presence as well as in the absence of PDC-109 at a protein/lipid ratio of 2.5 (w/w). In each case the spectrum obtained in the presence of protein is made up of two components. These two components have been resolved by spectral subtraction. Spectra obtained in the presence of PDC-109 and the subtraction results are shown in Fig. 1. In each set of four spectra the top pair correspond to the composite spectrum obtained in the presence of PDC-109 at 30°C (solid line) and a matching spectrum for the motionally restricted component alone (dotted line). The lower pair of spectra correspond to the fluid component obtained by subtracting the rigid component spectrum from the composite spectrum (solid line) and a matching fluid reference spectrum (dotted line). The fraction, *f*, of immobilized component obtained from spectral subtraction is also shown in the figure. The results indicate that presence of cholesterol has a significant effect on the size of the motionally restricted lipid component, the motionally restricted population being higher in the presence of cholesterol. This suggests that cholesterol potentiates the binding of PDC-109 to PC membranes. However, over the range of 5–20 wt% cholesterol, the motionally restricted fraction remains constant, or increases only slightly, within the range of error expected in the subtractions. Evidently, only relatively low concentrations of cholesterol are necessary to potentiate the PDC-109 binding.

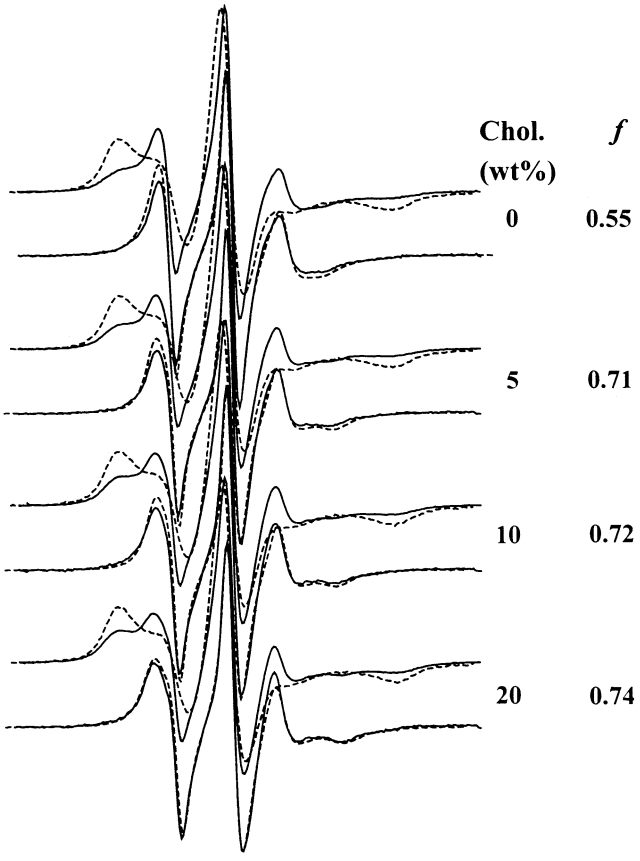

Fig. 1. Effect of cholesterol on the motionally restricted component of EPR spectra of 14-PCSL in membranes of DMPC and DMPC/cholesterol mixtures. In each set of four spectra, the upper pair corresponds to the spectrum obtained in the presence of PDC-109 at 30°C (solid line) and a matching spectrum for the motionally restricted component alone (dotted line). The lower pair corresponds to the fluid component obtained from spectral subtraction (solid line) and a matching fluid reference spectrum (dotted line). The fraction of cholesterol in the host membrane and the fraction of motionally restricted lipid component, *f*, obtained from spectral subtraction are indicated.

3.2. Lipid selectivity of PDC-109 in DMPC/cholesterol membranes

In further experiments, aimed at investigating the effect of cholesterol on the lipid selectivity, we have used fluid DMPC

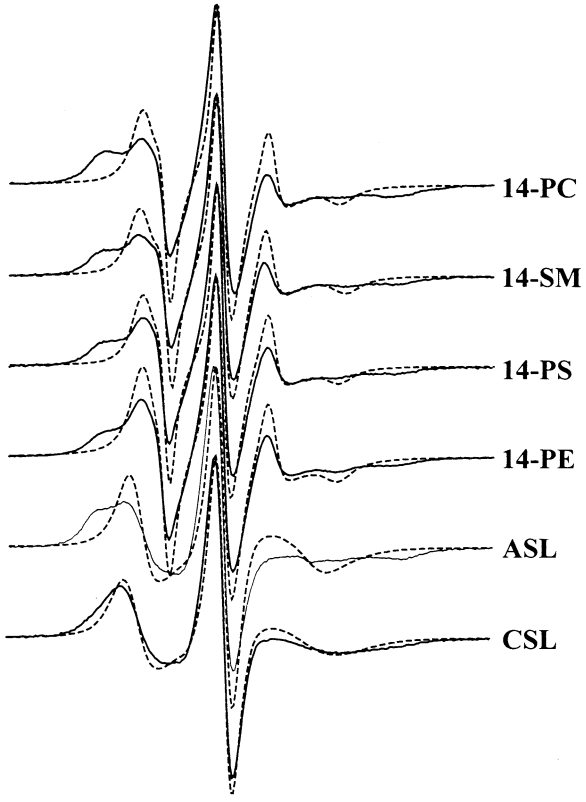

Fig. 2. EPR spectra of different phospholipid spin labels, 14-XXSL, bearing the spin label on the 14th C-atom of the *sn*-2 chain, as well as the cholestane spin label (CSL) and androstanol spin label (ASL), in DMPC membranes containing 20 wt% cholesterol. Spectra are shown for lipid mixture alone (dotted line) and in the presence of PDC-109 (lipid:protein, 1:2.5 w/w) (solid line). Phospholipid spin labels are: 14-PCSL, 14-SMSL, 14-PSSL and 14-PESL. Spectra were recorded at 30°C. The spectral width is 100 G.

membranes containing 20% (wt/wt) cholesterol as the host matrix, and 1 mol% of the desired spin-labelled lipid probe was incorporated into the lipid membranes. EPR spectra were recorded for the lipid samples alone and after addition of PDC-109 at a lipid/protein ratio of 1:2.5 (w/w). Representative spectra of different spin-labelled lipids in the absence and in the presence of PDC-109, recorded at 30°C, are given in Fig. 2. For all the spin labels investigated, the EPR spectra obtained in the presence of PDC-109 are composed of two components, as was the case with different spin-labelled lipids in DMPC membranes without cholesterol in the presence of PDC-109 [1]. The relative amounts of the two components in each spectrum are different, indicating the selectivity of the PDC-109 protein for different lipid species.

The two components in the EPR spectra of different spin-labelled lipid probes obtained in the presence of PDC-109 have been resolved by spectral subtractions and the relative contributions of each component to the overall spectrum have been quantified. The results obtained are shown in Fig. 3 for several of the spin labels. In each set of four spectra the top pair corresponds to the composite spectrum obtained in the presence of PDC-109 at 30°C (solid line) and a matching spectrum for the motionally restricted component alone (dotted line). The lower pair of spectra corresponds to the fluid component obtained by subtracting the motionally restricted component spectrum from the composite spectrum (solid line), and the spectrum of the same spin label, recorded in DMPC:cholesterol (80:20, w/w) at 30°C (dotted line). The relative amounts of the motionally restricted components (fraction, *f*) for the different spin-labelled lipids in the presence of PDC-109, obtained from the spectral subtractions, are given in Table 1. The effect of cholesterol on the specificity of PDC-109 for different lipid species and the corresponding lipid–protein stoichiometry is discussed below.

3.3. Effect of cholesterol on lipid stoichiometry and specificity

The equilibrium spin-label lipid–protein association can be described by (e.g. [19]):

$$\frac{1}{f}-1=\frac{1}{K_r}\left(\frac{n_t}{N_b}-1\right) \quad (1)$$

where f is the fraction of motionally restricted spin-labelled lipid, n_t is the ratio of lipid to bound protein, N_b is the number of lipid association sites on the protein and K_r is the association constant of the spin-labelled lipid relative to the host lipid. The association constant of a given spin-labelled lipid, relative to spin-labelled PC, is given by:

$$\frac{K_r}{K_r^{PC}}=\frac{1/f^{PC}-1}{1/f-1} \quad (2)$$

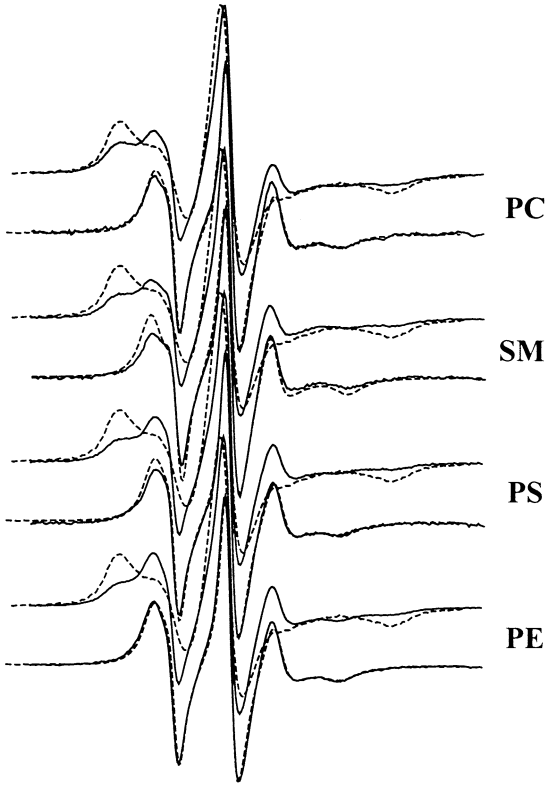

Fig. 3. Representative spectral subtractions for 14-position spin labels. Spectra of 14-PCSL, 14-SMSL, 14-PSSL and 14-PESL in DMPC/cholesterol (80:20, w/w) host matrix in the presence of PDC-109 (P/L weight ratio of 2.5) are shown. In each set of four spectra the upper pair corresponds to the composite spectrum of the PDC-109/lipid complex (solid line) and the motionally restricted component spectrum (dotted line) that is used for subtraction from the composite spectrum. The lower pair of spectra corresponds to the subtraction result (solid line) and a matching fluid lipid reference spectrum (dotted line). Spectra are all (including difference spectra) displayed normalized to the same maximum central line height.

Table 1
Effect of cholesterol on the selectivity and stoichiometry of lipid interactions with PDC-109 in complexes with DMPC

Spin label	Motionally restricted fraction (f)[a]	(K_r/K_r^{PC})[b]		$(n_t/N_b)/(n_t^o/N_b^o)$[c]
		+chol	−chol	
14-PCSL	0.74	1.0	1.0	0.64
14-SMSL	0.70	0.82	1.0	0.67
14-PSSL	0.64	0.63	0.61	0.63
14-PESL	0.58	0.49	0.34	0.59
ASL	0.59	0.51	0.61	0.67
CSL	0.42	0.25	–	–

See text for details.
[a]The f values are subject to an error of ca. ±0.02–0.05 due to uncertainties in the spectral subtractions. For CSL the motionally restricted component was too small to quantify reliably.
[b]Ratio of spin-labelled lipid association constant relative to that for 14-PCSL. Data for complexes with DMPC alone are from [1].
[c]Ratio of the lipid/protein-binding stoichiometry, n_t, to motionally restricted lipid stoichiometry, N_b, in the presence of cholesterol to that (viz., n_t^o/N_b^o) in the absence of cholesterol (i.e. for PDC-109/DMPC alone). See, e.g. [19].

This equation applies to the selectivity between different spin labels in the same protein–lipid complex, i.e. for constant n_t and N_b.

From the two-component spectra shown in Fig. 2 and the subtraction results given in Table 1, the selectivity of PDC-109 for different spin-labelled lipid probes in DMPC membranes containing 20 wt% cholesterol is in the following order: phosphatidylcholine ≥ sphingomyelin > phosphatidylserine > androstanol ≥ phosphatidylethanolamine ≫ cholestane. This trend is consistent with the lipid selectivity order observed for PDC-109 interacting with different lipid spin labels in a DMPC host matrix without cholesterol. With the exception of PE, it is seen from Table 1 also that the absolute values of the selectivity relative to PC, i.e. K_r/K_r^{PC}, are comparable for PDC-109 bound to DMPC membranes with and without cholesterol.

If it is thus assumed that the relative association constant, K_r, in the presence of cholesterol is equal to that in its absence, K_r^o, and further that $K_r^o = 1$ for spin-labelled PC, then:

$$\frac{n_t/N_b}{n_t^o/N_b^o} = f_o^{PC} + (1-f_o^{PC})\left(\frac{1/f-1}{1/f_o-1}\right) \quad (3)$$

where f_o and f_o^{PC} are the fractions of a given spin-labelled lipid and PC, respectively, that are motionally restricted in the absence of cholesterol. The left-hand side of Eq. 3 is the ratio of the lipid-binding stoichiometry to the intramembrane lipid/protein interaction stoichiometry, i.e. the value of n_t/N_b, relative to that in the absence of cholesterol. For spin-labelled PC, Eq. 3 reduces to:

$$\frac{n_t/N_b}{n_t^o/N_b^o} = \frac{f_o^{PC}}{f^{PC}} \quad (4)$$

If the unlabelled host lipid is PC, then Eq. 4 is the most direct means of determining the effect of cholesterol on the lipid/protein stoichiometries.

Values of $(n_t/N_b)/(n_t^o/N_b^o)$ calculated from Eq. 3, by using the present spin-label data for DMPC membranes with 20 wt% cholesterol and corresponding data of Ramakrishnan et al. [1] for DMPC membranes without cholesterol, are given in the final column of Table 1. With the exception of PE, the values are comparable for the different spin labels, consistent with the assumption that cholesterol does not have a large effect on lipid selectivity for PDC-109. As mentioned above, the value of $(n_t/N_b)/(n_t^o/N_b^o)$ for PC then most directly reflects cholesterol-induced changes in the lipid-binding stoichiometry. In principle, the decrease in the ratio n_t/N_b with cholesterol could be caused either by an increase in the number of lipid association sites, N_b, on PDC-109, or by an increase in binding stoichiometry, $1/n_t$, of PDC-109 to the membranes, in either case by approximately 50%. Most likely cholesterol potentiates the interaction between PC membranes and PDC-109 predominantly by the former mechanism, because fluorescence titrations have indicated no changes in binding stoichiometry at saturation by cholesterol addition [20].

Lipid-binding experiments have shown PDC-109 does not interact directly with cholesterol [5]. However, the results obtained in this study as well as our previous studies [1] demonstrate that the addition of PDC-109 to membranes containing PC and cholesterol results in an immobilization of the sterol, suggesting that PC probably mediates the interaction between PDC-109 and cholesterol. Here we show also that cholesterol potentiates the association of PDC-109 with lipids. This provides further insights into our understanding of the process of cholesterol efflux.

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