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ARTICLE

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Influence of the bovine seminal plasma protein PDC-109 on cholesterol in the presence of phospholipids

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Abstract The interaction of the major bovine seminal plasma protein PDC-109 with cholesterol was studied by employing spin-labelled analogues. It could be shown that PDC-109 does not interact directly with cholesterol molecules. However, in the presence of phospholipids we found a strong reduction of cholesterol motion by PDC-109. The fraction of immobilized cholesterol was largest for phosphorylcholine-containing lipids. This is consistent with the preferential interaction between PDC-109 and phosphatidylcholine. It is concluded that a stronger association and interaction of PDC-109 with phosphatidylcholine leads to an enhanced fraction of immobilized cholesterol analogues, but not to a phospholipid-dependent specific interaction between the protein and cholesterol. Moreover, the interaction of PDC-109 with various spin-labelled analogues of phosphatidylcholine (lysoPC, diacylPC) was investigated. In membranes of lipid vesicles the protein caused an immobilization of the phosphatidylcholine analogues mainly in the outer membrane leaflet, with no differences between diacylPC and lysoPC. The results are of relevance for understanding the physiological role of PDC-109 in the genesis of sperm cells.

Keywords Seminal plasma protein · PDC-109 · Cholesterol · ESR · Protein-lipid interaction

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Abbreviations *C5-SL-PC*: 1-palmitoyl-2-stearoyl(5-doxyl)-*sn*-glycero-3-phosphocholine · *cmc*: critical micellar concentration · *L*/*P*: molar ratio of lipid to protein · *LUV*: large unilamellar vesicle(s) · *lyso-PC*: lysophosphatidylcholine (1-palmitoyl-*sn*-glycero-3-phosphocholine) · *MCD*: β-methylcyclodextrin · *MLV*: multilamellar vesicle(s) · *PC*: phosphatidylcholine · *PE*: phosphatidylethanolamine · *PS*: phosphatidylserine · *sc-SL-PC*: short-chain spin-labelled phosphatidylcholine (1-palmitoyl-2-(4-doxylpentanoyl)-*sn*-glycero-3-phosphocholine) · *SL-A*: 3-doxyl-17β-hydroxy-5α-androstane · *SL-C*: 3β-doxyl-5α-cholestane · *SL-CHOL*: 25-doxylcholesterol · *SL-lysoPC*: spin-labelled lysoPC [1-stearoyl (16-doxyl)-*sn*-glycero-3-phosphocholine]

Introduction

PDC-109 is the major heparin-binding protein of bovine seminal plasma. It belongs to the family of seminal fibronectin type II (Fn 2) module proteins. Each of the members displays a mosaic architecture consisting of two tandemly arranged fibronectin type II domains and N-terminal distinctly *O*-glycosylated polypeptide extensions of various length. Fn2 module proteins have been also identified in equine (HSP-1/HSP-2) (Calvete et al. 1995) and porcine (Calvete et al. 1997) seminal plasma. Very recently, a novel group of proteins containing four Fn2 modules was cloned from human and canine epididymal cDNA libraries (Sallmann et al. 2001).

PDC-109, also denominated BSP-A1 and in its glycosylated isoform BSP-A2 (Esch et al. 1983), BSP-A3 (Seidah et al. 1987) and BSP-30K (Calvete et al. 1996a), are the major secretory products of the bovine seminal vesicles and bind to sperm upon ejaculation. It has been proposed that PDC-109 plays an important role in the capacitation process of spermatozoa, preparing them for the acrosome reaction. The main evidence for a physiological impact of the protein originated from studies of Manjunath and co-workers (Thérien et al. 1995), who showed that epididymal sperm cells undergo the acro-

some reaction more rapidly when preincubated with PDC-109. To this end, PDC-109 has to bind to the sperm cell plasma membrane (Aumüller et al. 1988; Manjunath et al. 1994a). According to Calvete et al. (1994), the high number of PDC-109 molecules bound per sperm cell suggests a multilayer coat of protein molecules around the cell.

In the course of the genesis of the sperm cell its plasma membrane is subject to an intensive remodeling in that the concentration, motional dynamics and structural organization of membrane constituents are changed (see Martínez and Morros 1996; Nolan and Hammerstedt 1997; Toshimori 1998). Several lines of evidence support the view that the modulating effect of PDC-109 on sperm cell capacitation arises from its interaction with membrane lipids. Firstly, it has been shown that PDC-109 specifically interacts with the phospholipid phosphatidylcholine (PC), suggesting that the protein interacts with spermatozoa by binding to this lipid on the sperm cell plasma membrane (Desnoyers and Manjunath 1992; Manjunath et al. 1994a; Gasset et al. 1997; Müller et al. 1998). This binding leads to an immobilization of lipids as found with spin-labelled lipid analogues (Müller et al. 1998; Greube et al. 2001). In this respect, the observation of changes of the mobility of membrane lipids, i.e. the membrane fluidity, during sperm cell capacitation is of particular interest (Wolf et al. 1996; Gadella et al. 1995). Secondly, PDC-109 affects phospholipid metabolism in sperm cells by inhibiting the activity of phospholipase A_2 (Manjunath et al. 1994b; Soubeyrand and Manjunath 1997). Phospholipase A_2 activity as well as the presence of lysolipids, especially lysophosphatidylcholine (lysoPC), are known to modulate capacitation and/or acrosome reaction (Fry et al. 1992; Llanos et al. 1993; Roldan and Fragio 1993a, 1993b; Riffo and Parraga 1997). Thirdly, efflux of cholesterol from the sperm cell membrane seems to be a crucial event for the process of capacitation (Davis 1981; Parks and Ehrenwald 1990; Benoff 1993). Indeed, several studies have shown that the sole extraction of cholesterol from sperm cells of different species by the cholesterol-binding substance β-methylcyclodextrin (MCD) is sufficient to capacitate those cells (Choi and Toyoda 1998; Visconti et al. 1999; Iborra et al. 2000). High-density lipoprotein as well as serum albumin have been already suggested to function as physiological extracellular acceptors for the cholesterol of sperm cells (Davis et al. 1980; Go and Wolf 1985; Langlais et al. 1988; Ehrenwald et al. 1990). However, recently it was found that PDC-109 extracts lipids, mainly PC and cholesterol, from the plasma membrane of epididymal sperm cells (Thérien et al. 1998, 1999) and of fibroblasts as a cell model (Moreau et al. 1999). Thus, very likely, PDC 109 may play an important role in cholesterol removal from the sperm plasma membrane.

The aim of the present study was to characterize the interaction of PDC-109 with cholesterol. In particular, we were interested whether PDC-109 interacts directly with cholesterol or whether an interaction is transmitted by other lipid components of the membrane. Recently, no indication for a direct binding of PDC-109 to cholesterol either solubilized in aqueous dispersion or linked to micro titer wells was found (Desnoyers and Manjunath 1992; Moreau and Manjunath 1999). This suggests that other lipid components might be involved in the interaction between PDC-109 and cholesterol. We surmise that PC lipids are essential for this interaction. Thus, we have studied the interaction of PC with PDC-109 and its relevance for the interaction between PDC-109 and cholesterol. Specific emphasis was given to lysoPC, which seems to play an important role during the capacitation of sperm cells (Ehrenwald et al. 1988; Roldan and Fragio 1993a).

For our purpose, we have employed spin-labelled analogues of steroids (androstane, cholestane and cholesterol) and of PC (Fig. 1) in conjunction with ESR spectroscopy. We found that, in the presence of PC (lysoPC, diacylPC), PDC-109 interacts with and causes an immobilization of spin-labelled analogues of steroids. In the absence of phospholipids, no interaction between the protein and steroid analogues was observed. In the presence of phosphatidylserine (PS) or

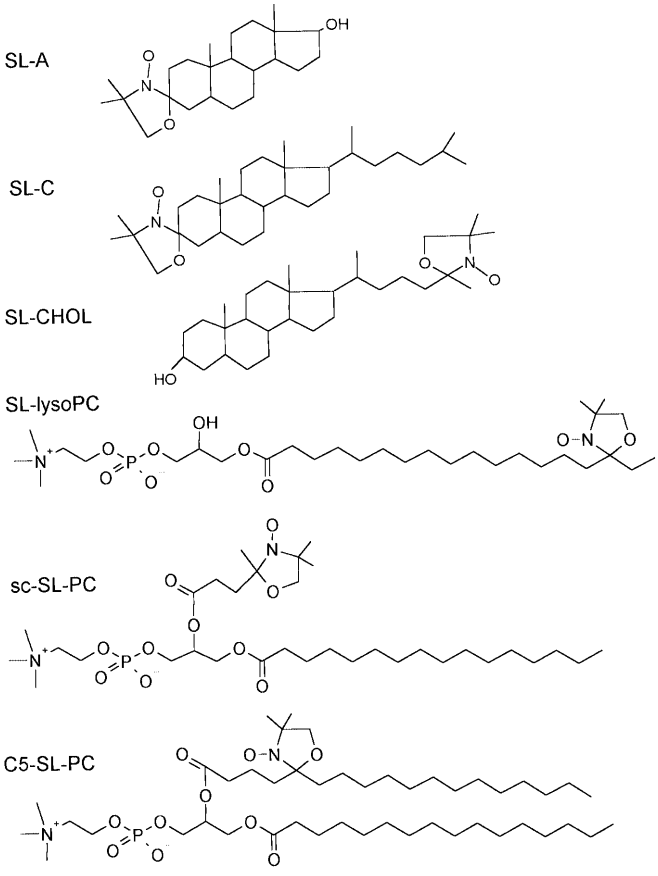

Fig. 1. Structure of the spin-labelled lipids used: 3β-doxyl-17 β-hydroxy-5α-androstane (SL-A), 3β-doxyl-5α-cholestane (SL-C), 25-doxyl-cholesterol (SL-CHOL), 1-stearoyl(16-doxyl)-*sn*-glycero-3-phosphocholine (SL-lysoPC), 1-palmitoyl-2-(4-doxylpentanoyl)-*sn*-glycero-3-phosphocholine (sc-SL-PC) and 1-palmitoyl-2-stearoyl(5-doxyl)-*sn*-glycero-3-phosphocholine (C5-SL-PC)

phosphatidylethanolamine (PE) the extent of immobilization of cholesterol was much less in comparison to PC. These results provide convincing evidence that an impact of PDC-109 on cholesterol requires the presence of PC lipids, which are the main component of the sperm cell plasma membrane.

Materials and methods

Chemicals

The structure of the spin-labelled lipids used is shown in Fig. 1. Spin-labelled analogues of cholesterol, 25-doxyl-cholesterol (SL-CHOL), and of lysoPC, 1-stearoyl(16-doxyl)-*sn*-glycero-3-phosphocholine (SL-lysoPC), were kindly provided by P.F. Devaux (Paris). Short-chain spin-labelled PC, 1-palmitoyl-2-(4-doxylpentanoyl)-*sn*-glycero-3-phosphocholine (sc-SL-PC), was prepared as previously described (Fellmann et al. 1994). Long-chain spin-labelled PC, 1-palmitoyl-2-stearoyl(5-doxyl)-*sn*-glycero-3-phosphocholine (C5-SL-PC), was from Avanti Polar Lipids (Alabama, USA). 3β-Doxyl-17β-hydroxy-5α-androstane (SL-A) and 3β-doxyl-5α-cholestane (SL-C) and all other chemicals, including lipids (PC and PE from egg, PS from brain), were purchased from Sigma (Deisenhofen, Germany).

Phosphate buffered saline (PBS) contained 150 mM NaCl and 5.8 mM Na_2HPO_4/Na_2HPO_4, pH 7.4. All incubations and measurements were performed at 4 °C if not stated otherwise.

Isolation of PDC-109

PDC-109 was purified from the seminal plasma of reproductively active Holstein bulls by combination of affinity chromatography on heparin-Sepharose and DEAE-Sephadex chromatography as described (Calvete et al. 1996b). The protein was pure as judged by SDS-polyacrylamide gel electrophoresis, reverse-phase HPLC analysis, N-terminal sequence, amino acid analysis and mass spectrometric analyses.

Incubation of PDC-109 with lipids in buffer or with labelled liposomes

The appropriate amount of the respective lipid(s) (labelled, unlabelled) in chloroform was added to a glass tube and the solvent was evaporated under nitrogen. Subsequently, PBS without or with PDC-109 (final concentration 1.3 mM) was added and the solution was mixed by pipetting. When using SL-A, SL-C or SL-CHOL, the solution was sonified to improve solubilization of the analogues (Branson sonifier 250, Danbury, USA; 1 min, duty cycle 20%, output control 2). The final lipid concentration was 25 μM and 2.5 mM for labelled and unlabelled lipids, respectively. After a 5 min incubation on ice, ESR measurements of the aqueous solutions were performed as described below.

For preparation of large unilamellar vesicles (LUV), PC and spin-labelled lipids (1 mol% of total lipids) dissolved in chloroform were combined in a glass tube. The mixture was dried under nitrogen. PBS was added to give a final lipid concentration of 5 mM and the lipids were hydrated by vigorous vortexing. LUV were prepared using an extruder (Lipex Biomembranes, Vancouver, Canada) with five freeze-thaw cycles and filtration through 0.1 μm pores (10 cycles) at 40 °C (Mayer et al. 1985). LUV (2.5 mM PC, 25 μM spin-labelled analogue) were incubated in the absence and in the presence of PDC-109 for 5 min on ice if not stated otherwise and, subsequently, ESR measurements were performed as described below.

Protein integrity at the respective experimental conditions was routinely checked by SDS-polyacrylamide electrophoresis (data not shown).

ESR measurements

ESR spectra of lipid solutions or LUV in the absence and in the presence of PDC-109 were recorded at 4 °C (if not stated otherwise) using a Bruker ECS 106 spectrometer (Bruker, Karlsruhe, Germany). Measuring parameters were as follows: modulation amplitude 4 G, power 20 mW, scan width 100 G, accumulation 16 times. Note that using a modulation amplitude of 4 G, signals of rapidly tumbling molecules are slightly overmodulated (see Fig. 5).

The ESR spectrum representing the immobilized analogue molecules was extracted by subtracting the ESR spectrum in the absence of protein from that in the presence of protein using standard ESR software (Bruker, Karlsruhe, Germany). From the double integrals of the spectra the fraction of immobilized lipids was estimated. The amount of the immobilized component is given as mean ± standard error of estimate, with n being the number of independent experiments. In case of two experiments, both the two individual values and the respective mean are presented.

Results

PDC-109 does not directly interact with spin-labelled analogues of steroids

The first set of experiments was aimed to investigate whether PDC-109 might directly interact with spin-labelled steroids. For that, we employed a model system (see Materials and methods) investigating (1) whether PDC-109 enhances the solubilization of steroids in aqueous buffer and (2) whether PDC-109 affects the mobility of solubilized steroids.

The spectrum of SL-A in aqueous solution indicates that this analogue was solubilized in buffer mainly as a monomer and that monomers underwent rapid tumbling, as seen from three narrow peaks (spectrum not shown). This can be rationalized from the structure of SL-A being rather hydrophilic with the doxyl group on carbon 3 and the absence of the hydrophobic tail on carbon 17 (see Fig. 1). The spectrum of SL-C in PBS consists of a broad peak which was caused by a strong spin-spin interaction due to the aggregation of this analogue in aqueous solution (spectrum not shown). The spectra of SL-A and SL-C after addition of PDC-109 (spectra not shown) are identical to those in the absence of the protein even at a rather high concentration of PDC-109 [molar ratio of lipid to protein (L/P) = 2]. This demonstrates that PDC-109 does neither interact with these analogues nor influence their mobility in aqueous solution.

Upon addition of buffer to a lipid film of SL-CHOL, no ESR signal was detected. However, after transferring the aqueous solution into a new tube and adding Triton X-100, a faint ESR spectrum consisting of three peaks was detected (not shown). The signal intensity of this spectrum measured by the double integral was about 1% of that of a control spectrum (analogue in chloroform). Triton X-100 is able to solubilize SL-CHOL since after addition of buffer containing the detergent to SL-CHOL we detected an intense ESR signal. From that we conclude that only a small amount of SL-CHOL was solubilized in PBS. Owing to an aggregation of the analogue in buffer the spin-spin interaction gives rise to a broad

ESR signal which is below the resolution of the ESR spectrometer. Addition of PDC-109 (L/P = 2) did not enhance the solubility of SL-CHOL. Moreover, in order to prove that SL-CHOL can be brought into solution at these conditions we employed MCD, which has been shown to solubilize cholesterol in aqueous solutions by incorporating cholesterol molecules into its hydrophobic ring (Klein et al. 1995; Yancey et al. 1996). Indeed, we found an efficient solubilization of SL-CHOL by MCD, as indicated by an intense ESR spectrum of the analogue (not shown). For example, at 10 mM MCD the signal intensity of the spectrum was about 60% of that of the control spectrum (SL-CHOL in chloroform). Taken together, these data strongly support that PDC-109 does not interact directly with cholesterol.

Influence of PDC-109 on spin-labelled cholesterol is mediated by PC

SL-CHOL can be solubilized in PBS in the presence of phospholipids, as concluded from the respective ESR spectra. In Fig. 2A-I the ESR spectrum of SL-CHOL in aqueous dispersion with lysoPC is given, showing that (part of) the cholesterol analogue was solubilized at this condition. By comparison with the control spectrum of the analogue in chloroform, we estimated that about 12% of SL-CHOL became solubilized. It can be surmised that the lipid mixture of lysoPC and SL-CHOL is organized in the aqueous buffer mainly as micelles, since the concentration of lysoPC was above its critical micellar concentration (cmc). The shape of the spectrum of Fig. 2A-I reflects some restriction of rapid motion of SL-CHOL in those micelles. A second spectral component arose in the presence of PDC-109 (L/P = 2) (see arrows in Fig. 2A-II). This new component indicated a decreased mobility of the analogue in the presence of PDC-109. The amount of spin-labelled analogues was similar to that in the absence of the protein, as deduced from double integration of spectra. This indicates that although PDC-109 caused immobilization of the analogue in the presence of phospholipids, it does not facilitate the solubilization of cholesterol in the aqueous dispersed lipid phase. Double integration of the protein-mediated immobilized component (Fig. 2A-III) was obtained by subtraction of the ESR spectrum in the absence of PDC-109 from that in the presence of the protein and revealed that about 76% ($n = 2$, 72% and 79%) of SL-CHOL was affected by the protein.

PC solubilized in aqueous buffers forms bilayers, mainly organized as multilamellar vesicles (MLV). The ESR spectrum of SL-CHOL in aqueous dispersion with PC reflects a typical membrane spectrum, indicating that SL-CHOL was incorporated into the membrane of those MLV (Fig. 2B-I). About 40% of SL-CHOL became solubilized in PC-MLV under those conditions. This amount of solubilized SL-CHOL was not enhanced in the presence of PDC-109. However, in the presence of PDC-109 (L/P = 2) a second, more immobilized component of SL-CHOL was observed, indicating a more restricted mobility of a fraction of analogue molecules (Fig. 2B-II, see arrows). The immobilized component (Fig. 2B-III) corresponded to $57.7 \pm 5.8\%$ ($n = 3$) of analogue molecules.

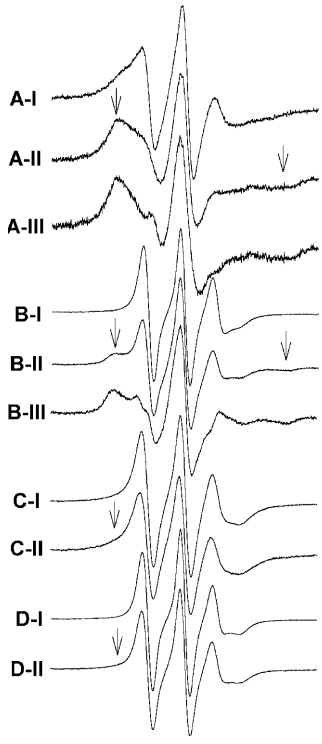

Fig. 2. Influence of PDC-109 on the ESR spectrum of the aqueous solution of spin-labelled cholesterol (SL-CHOL) in the presence of phospholipids. The organic solution of SL-CHOL and lysoPC (**A**), PC (**B**), PS (**C**) or PC/PE (1:1) (**D**) was mixed in a glass tube and the solvent was evaporated. Subsequently, PBS without (spectra **I**) or with PDC-109 (final concentration 1.3 mM) (spectra **II**) was added to give final lipid concentrations of 25 μM and 2.5 mM for SL-CHOL and the respective phospholipid. After a 5 min incubation on ice, ESR measurements were performed as described in Materials and methods. The immobilized component in the presence of the protein (see *arrows*) was obtained by spectral subtraction, yielding spectra **III**

These data suggest that the mobility of the spin-labelled analogue of cholesterol is affected by PDC-109 in a lipidic environment. In order to test whether solubilization of SL-CHOL is solely mediated by the respective phospholipid head group, a solution of glycerophosphorylcholine chloride without or with PDC-109 was added to the dried analogue. After resuspension, in both cases, i.e. in the absence and in the presence of the protein, no ESR signal was detected, supporting the role of the fatty acid chains and the hydrophobic lipid phase for the solubilization of SL-CHOL and its interaction with PDC-109.

Immobilization of spin-labelled cholesterol depends on the phospholipid head group

In a further set of experiments we investigated whether the immobilization of SL-CHOL in lipid bilayers caused by

PDC-109 is dependent on the phospholipid head group. SL-CHOL could be solubilized in the presence of PS or PC/PE, as seen from the ESR spectra shown in Fig. 2C-I and Fig. 2D-I, respectively. We estimated that about 46% and 43% of SL-CHOL was solubilized in the presence of PS and PC/PE, respectively. The spectra in the presence of the protein display the occurrence of an additional more immobilized component (Fig. 2C-II and Fig. 2D-II, arrows). However, the extent of immobilization was much less pronounced for PS and PC/PE, as found for PC without other phospholipids (see Fig. 2B-II). These results show that the effect of PDC-109 on cholesterol is dependent on the phospholipid head group and underline that PC is required for an efficient influence of the protein on SL-CHOL. The amount of recovered SL-CHOL in the phospholipid phase was almost independent of the phospholipid used here.

Influence of PDC-109 on spin-labelled cholesterol in LUV membranes

To address whether PDC-109 might influence SL-CHOL when interacting with a preformed membrane, ESR spectra of SL-CHOL labelled PC-LUV were recorded in the absence and in the presence of the protein (L/P = 2) (Fig. 3). The spectra reveal that PDC-109 also affected cholesterol within a membrane in that an immobilized component occurred subsequent to the addition of the protein (Fig. 3B). From spectral subtraction it was estimated that $20.5 \pm 5.1\%$ ($n = 4$) of analogue molecules were affected by the protein. The immobilization degree of SL-CHOL was similar at a L/P of 2 and 10 and became lower at L/P = 20 (Fig. 4). Thus, as expected, PDC-109 also mediates an immobilization of SL-CHOL upon interaction with membranes of labelled liposomes. Importantly, the immobilized fraction of SL-CHOL was not enhanced in the presence of 5 or 10 mol% lysoPC in the LUV membranes (not shown).

Further, the influence of PDC-109 on LUV labelled with SL-A and SL-C, respectively, was investigated. We found that also these steroid analogues were immobilized by the protein, as deduced from the ESR spectra in the absence and in the presence of the protein. However, the degree of protein-mediated immobilization was larger compared with that of SL-CHOL in that the immobilized component (see Fig. 3D and E) represented $34.7 \pm 1.8\%$ ($n = 3$) and $49.7 \pm 2.3\%$ ($n = 3$) of SL-C and SL-A, respectively.

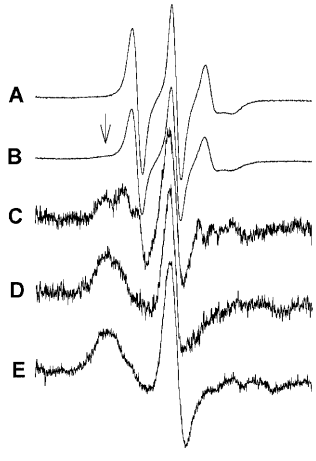

Fig. 3. Influence of PDC-109 on the ESR spectrum of spin-labelled cholesterol (SL-CHOL) incorporated into PC-LUV membranes. 2.5 mM PC-LUV were labelled with 25 μM SL-CHOL and the ESR spectra were recorded in the absence (**A**) and in the presence (**B**) of 1.3 mM PDC-109 as described in Materials and methods. In the latter case a strongly immobilized component was observed (see *arrow*), which was obtained by spectral subtraction, yielding spectrum **C**. **D**, **E** are the respective spectra of the immobilized component obtained from PC-LUV labelled with spin-labelled cholestane and androstane, respectively, in the presence of PDC-109

Influence of PDC-109 on spin-labelled analogues of PC

The results obtained above indicate not only an influence of the phospholipid head group on the interaction of PDC-109 with cholesterol but also of other structural properties of the phospholipids. As shown above, the interaction of PDC-109 with SL-CHOL was more pronounced in the presence of lysoPC compared with diacylPC. To address this observation further, we have investigated the interaction of PDC-109 with various spin-labelled analogues of PC (SL-lysoPC, sc-SL-PC and C5-SL-PC). Previously, we have shown that PDC-109 decreases the mobility of spin-labelled PC in membranes of liposomes (Müller et al. 1998; Greube et al. 2001).

To show first whether PDC-109 interacts with lysoPC, we studied the interaction of the protein with SL-lysoPC in the absence of other lipids. The ESR

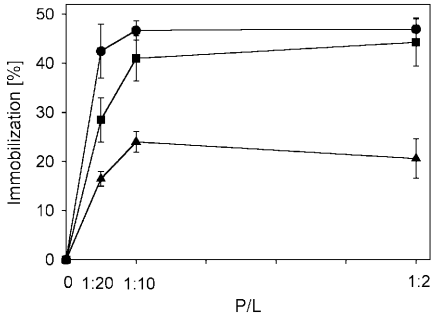

Fig. 4. Influence of PDC-109 on the immobilization degree of spin-labelled lipids incorporated into PC-LUV membranes. 2.5 mM PC-LUV were labelled with 25 μM SL-CHOL (*triangles*), C5-SL-PC (*squares*) or sc-SL-PC (*circles*) and the ESR spectra were recorded in the absence and in the presence of different concentrations of PDC-109, yielding different molar ratios of protein to lipid (P/L). The amount of immobilized lipids in the presence of the protein was estimated by spectral subtraction (see Materials and methods). Data are given as mean ± standard error of estimate of at least three independent experiments

spectrum of SL-lysoPC dissolved in PBS is shown in Fig. 5A-I. Under these conditions, SL-lysoPC was mainly organized as monomers since we could not observe a broad line of spin-spin interaction which would be expected for analogues organized in micelles. The isotropic spectrum of the aqueous solution of SL-lysoPC indicates a rapid tumbling of the analogue monomers (Fig. 5A-I). Upon resuspension of SL-lysoPC in the presence of PDC-109, the ESR spectrum consists of two components (Fig. 5A-II), one isotropic arising from analogue monomers. The second component consists of a very broad signal (see arrows in Fig. 5A-II) and is indicative for a spin-spin interaction of the analogue. Very likely, this was caused by binding of more than one analogue molecule to a protein molecule. The close apposition of analogues will result in a line broadening of ESR signals. To prevent such a local enrichment of analogue molecules, SL-lysoPC was mixed with an excess of (unlabelled) lysoPC. Note that the concentration used was above the cmc of lysoPC (see above). Therefore, the lipids are expected to be organized as monomers and micelles, which is confirmed from the respective ESR spectrum (Fig. 5B-I). The spectrum consists of two components. The more immobile component (see arrows in Fig. 5B-I) corresponds to SL-lysoPC in micelles with (unlabelled) lysoPC, whereas the mobile one reflects analogue monomers (see arrow heads in Fig. 5B-I). After solubilization of lysoPC and SL-lysoPC in a PDC-109-containing buffer (L/P = 2), the ESR spectrum reflected a restriction of the mobility of nearly all SL-lysoPC (Fig. 5B-II), indicating a strong interaction of PDC-109 with lysoPC.

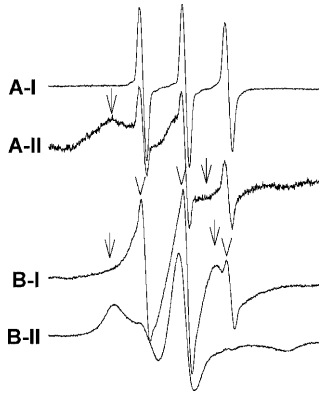

Fig. 5. Influence of PDC-109 on the ESR spectrum of the aqueous solution of spin-labelled lysoPC (SL-lysoPC) in the absence and in the presence of non-labelled lysoPC. The organic solution of SL-lysoPC without (**A**) and with non-labelled lysoPC (**B**) was added to a glass tube and the solvent was evaporated. Subsequently, PBS without (spectra **I**) or with PDC-109 (final concentration 1.3 mM) (spectra **II**) was added to give a final lipid concentration of 25 μM SL-lysoPC (**A**) or of 25 μM SL-lysoPC and 2.5 mM lysoPC (**B**). After a 5 min incubation on ice, ESR measurements were performed as described in Materials and methods. In spectrum **A-II** the *arrows* denote a broad signal arising in the presence of the protein. In spectrum **B-I** the peaks of the free tumbling label monomers (*arrowheads*) and of the label organized in micelles (*arrows*) are denoted

ESR spectra of the suspension of PC and spin-labelled PC analogues (SL-lysoPC, sc-SL-PC or C5-SL-PC) indicate that the analogues were incorporated into membranes of PC-MLV (Fig. 6, spectra I). Although C5-SL-PC and sc-SL-PC bear the label moiety at the *sn*-2 position close to the head group, the spectrum of C5-SL-PC reflects a lower mobility compared with sc-SL-PC owing to the longer fatty acid chain of C5-SL-PC. On suspending PC analogues and PC in the presence of PDC-109 (L/P = 2), a second, more immobilized component was observed (Fig. 6, spectra II). From spectral subtraction (Fig. 6, spectra III) we estimated that 81% ($n = 2$, 79%, 83%), 69% ($n = 2$, 62%, 76%) and 62% ($n = 2$, 58%, 66%) of SL-lysoPC, sc-SL-PC and C5-SL-PC, respectively, were immobilized by PDC-109. The extent of immobilization may correspond to the critical concentration of the lipids to form supramolecular structures (micelles, bilayers) rather than to reflect a specific lipid interaction of PDC-109. During this mode of incubation, lipids such as SL-lysoPC may become more easily suspended in buffer and, thus, may compete efficiently for binding sites of PDC-109 with respect to diacylPC.

To elucidate whether a preferred interaction of PDC-109 with SL-lysoPC can be also observed when the protein interacts with lipids after membrane formation, we compared the interaction of PDC-109 with

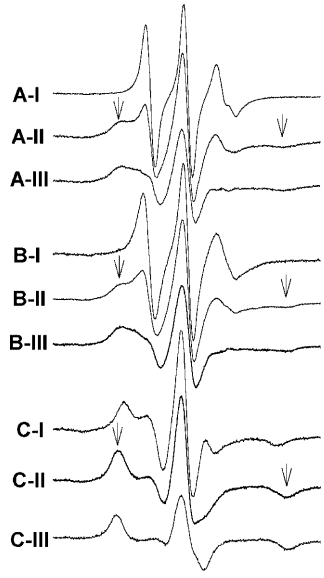

Fig. 6. Influence of PDC-109 on the ESR spectrum of the aqueous solution of spin-labelled PC lipids in the presence of PC. The organic solution of PC and SL-lysoPC (**A**), sc-SL-PC (**B**) or C5-SL-PC (**C**) was mixed in a glass tube and the solvent was evaporated. Subsequently, PBS without (spectra **I**) or with PDC-109 (final concentration 1.3 mM) (spectra **II**) was added to give final lipid concentrations of 25 μM and 2.5 mM for the respective spin-labelled analogue and PC. After a 5 min incubation on ice, ESR measurements were performed as described in Materials and methods. The immobilized component in the presence of the protein (see *arrows*) was extracted by spectral subtraction, yielding spectra III

SL-lysoPC, sc-SL-PC and C5-SL-PC, respectively, incorporated into PC-LUV. We estimated that 47% ($n=2$, 44%, 50%), $47.1 \pm 2.0\%$ ($n=4$) and $44.3 \pm 4.9\%$ ($n=3$) of SL-lysoPC, sc-SL-PC and C5-SL-PC, respectively, were immobilized by the protein (L/P = 2) suggesting that (1) the interaction of PDC-109 is similar for diacylPC and lysoPC in lipid bilayers and (2) mainly the analogue molecules in the outer membrane leaflet are affected. To give further support for the latter point, we measured the PDC-109 mediated immobilization of sc-SL-PC and C5-SL-PC in PC-LUV at 25 °C. The degrees of immobilization also argue for a main impact of the protein on the outer membrane leaflet (sc-SL-PC: 56%, $n=2$, 57%, 55%; C5-SL-PC: $44.0 \pm 3.5\%$, $n=4$).

Moreover, we have investigated the effect of PDC-109 on sc-SL-PC and C5-SL-PC in LUV membranes at higher, more physiologically relevant L/P (Fig. 4). The degree of analogue immobilization found at a L/P of 10 was similar to that obtained at L/P = 2. At higher L/P (20), less analogues were affected in mobility by the protein.

Discussion

The emphasis of the present study was to characterize the interaction of the bovine seminal plasma protein PDC-109 with cholesterol. It has been shown that PDC-109 is able to extract cholesterol and PC from the plasma membrane of sperm cells (Thérien et al. 1998, 1999), which is considered to resemble an important step in capacitation of sperm cells (Davis 1981; Parks and Ehrenwald 1990; Benoff 1993). With this regard, the investigation of cholesterol interaction with PDC-109 is of great relevance. In order to identify modes of interaction, in particular direct protein-lipid interactions, we have studied in part of the experiments the suspension of lipids in buffer in the presence of rather high PDC-109 concentrations. By employing this model approach and various spin-labelled analogues, we could unambiguously demonstrate that PDC-109 does not interact directly with steroid molecules, in particular with cholesterol. For example, even in the presence of PDC-109, no significant amounts of the spin-labelled analogue SL-CHOL were taken up into the aqueous medium. From that we can definitely exclude a direct interaction of PDC-109 with cholesterol, leading to its extraction. Even those steroid analogues of cholestane and androstane which became suspended in buffer in the absence of PDC-109, and other lipids, did not bind to PDC-109. The lack of a direct interaction between PDC-109 and cholesterol supports previous suggestions (Desnoyers and Manjunath 1992; Moreau and Manjunath 1999).

In the presence of phospholipids we found a significant uptake of SL-CHOL into the aqueous buffer phase. As deduced from the ESR spectra, SL-CHOL becomes organized in the phospholipid phase. A strong immobilized fraction of the analogue was observed in the presence of PDC-109. This effect was specifically dependent on lipid head groups. In the presence of phospholipids having the phosphorylcholine head group (PC), SL-CHOL exhibits a much stronger interaction with PDC-109 in comparison to other phospholipids such as PS or PE, for which a considerably weaker immobilization was detected. We surmise that the larger fraction of immobilized SL-CHOL is a result of the preferred direct interaction of PDC-109 with PC in comparison with the two aminophospholipids. This preference of PDC-109-phospholipid interaction has been demonstrated previously by various studies (Desnoyers and Manjunath 1992; Manjunath et al. 1994a; Gasset et al. 1997; Müller et al. 1998). The stronger association and interaction of PDC-109 with PC bilayers leads to an enhanced fraction of immobilized cholesterol analogues, but not to a phospholipid-dependent specific interaction between the protein and cholesterol (see below).

From our measurements, no conclusions about the molecular structure of the lipid-protein complexes can be drawn. In aqueous buffer, PDC-109 tends to aggregate, depending on the solution composition (Gasset et al. 1997). At our experimental conditions the protein is expected to be organized as monomers and dimers (Gasset et al. 1997). The process of binding to lipids which also might induce a dissociation of oligomers involves changes of the protein conformation (Müller et al. 1998; Gasset et al. 2000). Some studies have shown that, upon interaction of PDC-109 with lipids, lipid-protein particles are formed which have been compared structurally with high-density lipoproteins (Moreau and Manjunath 1999; Ramakrishnan et al. 2001).

PDC-109 also caused an immobilization of SL-CHOL in membranes of PC-LUV. Under our conditions, we observed that about 20% of the SL-CHOL became immobilized by PDC-109. This is much less in comparison to the fraction of immobilized analogues of lysoPC and diacylPC in PC-LUV. In that case, those analogues were immobilized by the protein to an extent of about 50%, indicating that, very likely, all analogues of the outer membrane leaflet were affected by PDC-109. Also at the higher L/P of 10 a similar amount of spin-labelled diacylPC was affected by PDC-109. Previously, we have shown that the LUV membrane remains intact at these conditions (Greube et al. 2001). The immobilization of sc-SL-PC and C5-SL-PC at different protein concentrations suggests a stoichiometry of PC/PDC-109 interaction of about 10, which is very similar to the results of other studies (Müller et al. 1998; Gasset et al. 2000; Ramakrishnan et al. 2001) and agrees with volumetric estimates (Ramakrishnan et al. 2001).

We surmise that the lower fraction of immobilized SL-CHOL in PC-LUV supports the above view in that cholesterol is not directly affected by PDC-109. The question remains of how cholesterol could be partially immobilized in a fully immobilized phospholipid phase. According to its structure, cholesterol, being predominantly of hydrophobic nature, penetrates deeply into the

hydrophobic part of the membrane. While phospholipids are anchored to the respective leaflet of a bilayer by their large polar head groups, those polar elements are lacking in cholesterol. It has been shown previously that cholesterol exhibits a dynamic, fluctuating motion parallel to the membrane normal, thereby protruding into the opposite monolayer of the bilayer (Huster et al. 1998; Gliss et al. 1999). We propose that upon binding of PDC-109 to a SL-CHOL/PC-containing membrane, the PC molecules in the outer membrane leaflet become highly restricted in their mobility and the cholesterol analogues sense the impact of the protein indirectly and to a smaller extent in comparison to PC (1) owing to the absence of a specific interaction with PDC-109 and (2) owing to the cholesterol dynamics in membranes.

The presence of (unlabelled) lysoPC in PC-LUV did not enhance the fraction of immobilized SL-CHOL. Thus, although we observed a very large fraction of immobilized SL-CHOL when suspended in buffer in the presence of lysoPC and PDC-109, we have no indication that the interaction with cholesterol becomes enhanced when PDC-109 is added to lysoPC-containing membranes. This is in line with the observation that the interaction of PDC-109 with SL-lysoPC in LUV membranes is similar to that with spin-labelled diacylPC, i.e. although we found under certain circumstances a preferred interaction between SL-lysoPC and PDC-109 with respect to diacylated PC analogues (sc-SL-PC and C5-SL-PC) (see results), we have no evidence for such a preference when PDC-109 is added to membranes containing the respective PC analogues. However, this conclusion may require a critical consideration (see last paragraph).

We also found an interaction of PDC-109 with other steroid molecules, i.e. androstane and cholestane, under those conditions. While in aqueous dispersions no effect of PDC-109 on these molecules was observed, the protein caused an immobilization of SL-A and SL-C in LUV membranes. The extent of immobilization of spin-labelled steroids increased in the order SL-CHOL, SL-C, SL-A. This is in line with the results of Ramakrishnan et al. (2001), who also described a higher immobilization of spin-labelled androstane compared to spin-labelled cholestane by hydrating the lipid films of dimyristoylPC and the respective analogue directly with PDC-109, similar to the model system used here. The differences between the analogues suggest that the effect of PDC-109 on steroids in the presence of PC depends on the structure of the steroid molecule. We note that the analogue SL-CHOL is a trustworthy reporter of endogenous cholesterol, while SL-C and SL-A may differ in their properties with respect to cholesterol (Morrot et al. 1987; P. Müller and A. Herrmann, unpublished results). In the bull sperm cell, part of cholesterol is esterified at C3 (Kelso et al. 1997). SL-A and SL-C are also lacking the OH group at C3 bearing the label moiety at this carbon (see Fig. 1).

The results of this study are of relevance for understanding the role of PDC-109 in the genesis of sperm cells. Firstly, we could show that the binding of PDC-109 to membranes causes an immobilization of PC and cholesterol, the major lipid components of the exoplasmic leaflet of the sperm plasma membrane. This protein-mediated rigidification of the plasma membrane might be one of the factors which prevent sperm cells from an inappropriate acrosome reaction during their passage through the female reproductive tract (see Martínez and Morros 1996; Nolan and Hammerstedt 1997). Secondly, the effect of PDC-109 on steroids in the presence of PC presents a basis for understanding the protein-mediated extraction of lipids (PC, cholesterol) from membranes (Thérien et al. 1998; Moreau et al. 1999). While the release of PC from membranes could be rationalized since PDC-109 has been shown to specifically bind/interact to/with this lipid (Desnoyers and Manjunath 1992; Manjunath et al. 1994a; Gasset et al. 1997; Müller et al. 1998), so far no evidence has been given for a mechanism giving PDC-109 access to cholesterol. Thirdly, upon short-term incubation with PDC-109 we did not find qualitative differences for the protein-lipid interaction between the various spin-labelled PC analogues, including that of lysoPC. However, directing future studies, we cannot preclude at the present state of investigation that the specific composition of the sperm cell membrane may support a preferred interaction, e.g. between lysoPC and PDC-109. Those studies are currently being undertaken. In particular, a topic of future research should be the protein-lipid interaction upon prolonged incubation with PDC-109 and the characterization of released PDC-109-lipid complexes. Manjunath and co-workers (Moreau et al. 1999) described a PDC-109-mediated extraction of lipids, e.g. from fibroblasts at 37 °C with a half time of about 10 min. Here, we observed the protein-mediated immobilized component in the ESR spectra of SL-CHOL within one minute after mixing protein and LUV. Moreover, protein-membrane interaction was investigated at a low temperature (4 °C). Therefore, we surmise that our results reflect the interaction between PDC-109 and spin-labelled lipids located in membranes. Furthermore, it is interesting that PDC-109 inhibits the activity of phospholipase A_2 (Manjunath et al. 1994b; Soubeyrand and Manjunath 1997), which has been proposed to play an important role during capacitation and acrosome reaction of sperm cells (Fry et al. 1992; Roldan and Fragio 1993a; Riffo and Parraga 1997). Therefore, it would extremely important to address whether changes in the activity of phospholipase A_2 influence the amount of lysolipids in the membrane, which in turn could modify the interaction of PDC-109 with the sperm cell membrane.

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