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ARTICLE

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Biophysical characterization of the interaction of bovine seminal plasma protein PDC-109 with phospholipid vesicles

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Abstract PDC-109 is the major protein of bovine seminal plasma. It binds to the bovine sperm surface at ejaculation and modulates sperm capacitation. PDC-109 displays phosphorylcholine- and heparin-binding activities which are thought to account for its sperm surface coating and glycosaminoglycan-induced sperm capacitating activities, respectively. We have characterized the interaction of isolated PDC-109 with membranes of phospholipid vesicles using a biophysical approach. Our results show that PDC-109 interacts not only with the solvent-exposed phosphorylcholine head group but also with the hydrophobic core of liposomes. Binding of PDC-109 to membranes is a very rapid, biphasic process with half times of less than one second. Maximal binding of PDC-109 to small unilamellar vesicles was achieved with a stoichiometric ratio of 10–11 phosphatidylcholine molecules/PDC-109 molecule. Incorporation of phosphatidylethanolamine or phosphatidylserine into phosphatidylcholine vesicles reduced the binding of PDC-109, suggesting that both the density of phosphorylcholine groups and the surface charge determine the interaction of the seminal plasma protein with the surface of the membrane. Electron spin resonance measurements showed that binding of PDC-109 to phosphatidylcholine vesicles caused a rigidification of the membrane. The relevance of the data for describing the role of PDC-109 in the modulation of sperm capacitation is discussed.

Key words Seminal plasma protein · PDC-109 · Membrane · Fluorescence · ESR

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Abbreviations *2, 12AS* 2-, 12-(9-anthroyloxy)stearic acid · *dansylPE* (N-(5-dimethylaminonaphthalene-1-sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine · *ESR* Electron spin resonance · *lysoPC* L-α-lysophosphatidylcholine · *lysoPS* L-α-lysophosphatidyl-L-serine · *PBS* Phosphate buffered saline · *PC* L-α-phosphatidylcholine · *PE* L-α-phosphatidylethanolamine · *PS* L-α-phosphatidylserine · *SL-PC* Spin-labelled PC (1-palmitoyl-2-(4-doxyl-pentanoyl)-PC) · *SL-PE* Spin-labelled PE (1-palmitoyl-2-(4-doxyl-pentanoyl)-PE) · *SM* Sphingomyelin · *SUV* Small unilamellar vesicles

Introduction

Seminal plasma, the fluid in which mammalian spermatozoa are suspended in semen, is made up by the secretions of the male accessory reproductive organs and appears to exert important effects on sperm function (Shivaji et al. 1990). Thus, the seminal plasma of a variety of mammalian species (including pig, cat, rat, goat, horse, ram, bull, rabbit, and human) contains decapacitation factors which prevent inappropriate acrosome reactions (Chang 1957). Inactivation or release of these factors might modulate capacitation *in vivo* during sperm residence in the female's genital tract, thereby enhancing the fertilizing capacity of spermatozoa. In addition, heparin-binding proteins of bovine seminal plasma bind to phosphorylcholine-containing lipids of cauda epididymal spermatozoa at ejaculation. The interaction of these proteins with heparin-like glycosaminoglycans secreted by the epithelium of the female reproduction tract enhance the fertilizing capacity of spermatozoa (Thérien et al. 1995).

*B*ovine *s*eminal *p*lasma contains four major acidic proteins, designated as BSP-A1, BSP-A2, BSP-A3, and BSP-30K (Manjunath et al. 1987; Chandonnet et al. 1990). The primary structures of these proteins have been reported (Esch et al. 1983; Seidah et al. 1987; Calvete et al. 1996a). BSP-1, also termed PDC-109 (Esch et al. 1983) and Major Protein (Scheit et al. 1988), has the same polypeptide chain as BSP-A2 but is O-glycosylated (Calvete et al.

1994; Gerwig et al. 1996). BSP-A1/A2, BSP-A3, and BSP-30K possess a mosaic structure made up of N-terminal extensions of variable length and containing nonconserved O-glycosylation sites followed by two homologous domains each displaying the consensus sequence of the fibronectin type II module (Calvete et al. 1996a). These proteins are secretory products of the seminal vesicles (Manjunath et al. 1987; Scheit et al. 1988) and bind to spermatozoa upon ejaculation (Aumüller et al. 1988; Manjunath et al. 1994). The BSP proteins also bind to heparin (Chandonnet et al. 1990) and, when the purified proteins were coincubated with bovine epididymal sperm, all of them invoked synergistically and in a concentration-dependent manner in vitro sperm capacitation stimulated by heparin, as measured by the onset of acrosome reactions upon exposure to lysophosphatidylcholine (lysoPC) (Thérien et al. 1995). Bovine epididymal sperm undergo the lysoPC-induced acrosome reaction only in the presence of BSP proteins and the BSP proteins cannot stimulate the acrosome reaction in the absence of heparin (Thérien et al. 1995). The mechanism by which these bovine seminal plasma proteins modulate sperm capacitation is unknown. It is noteworthy that PDC-109 interacts with a number of ligands other than heparin, including collagen, fibrinogen, apolipoprotein (apo)A1, as well as apoA1 associated with high-density lipoprotein (HDL) (Manjunath et al. 1988; Manjunath et al. 1989; Chandonnet et al. 1990). This suggests that PDC-109 could recruit other factors to the capacitating sperm surface.

The binding sites of the four BSP proteins on the sperm surface appear to be lipids: BSP-A1, BSP-A2, and BSP-A3 bind specifically to phospholipids which contain the phosphorylcholine (PC) group (Desnoyers and Manjunath 1992). Around 9.5 million PDC-109 molecules bind to the surface of a bull spermatozoon upon ejaculation and this figure decreases only slightly (7.7×10^6 molecules per cell) following in vitro capacitation (Calvete et al. 1994). BSP-30K displays a much broader binding specificity. It preferentially binds to choline phospholipids but also interacts with phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol, phosphatidic acid, and cardiolipin (Desnoyers and Manjunath 1992).

In the present study we used spectroscopic methods (electron spin resonance (ESR), fluorescence) to investigate, directly and quantitatively, the interaction of PDC-109 with liposomal membranes. In addition, stopped-flow measurements were used to study the kinetics of the binding of PDC-109 to liposomal membranes.

Materials and methods

Chemicals

Phospholipids: L-α-phosphatidylcholine (PC), L-α-phosphatidylethanolamine (PE), L-α-phosphatidylserine (PS), sphingomyelin (SM); lysolipids: L-α-lysophosphatidylcholine (lysoPC), L-α-lysophosphatidyl-L-serine (lysoPS); and phospholipid head groups: O-phosphorylethanolamine, phosphorylcholine chloride were purchased from Sigma (Deisenhofen, Germany). (N-(5-dimethylaminonaphtalene-1-sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (dansylPE) and 2-, 12-(9-anthroyloxy)-stearic acids (2, 12AS) were from Molecular Probes (Oregon, USA). Spin-labelled phospholipids: 1-palmitoyl-2-(4-doxyl-pentanoyl)-phosphatidylcholine (SL-PC), and -phosphatidylethanolamine (SL-PE) were prepared as described previously (Fellmann et al. 1994).

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. If not stated otherwise, measurements were performed in phosphate buffered saline (PBS) containing 150 mM NaCl and 5.8 mM Na_2HPO_4/NaH_2PO_4, pH 7.4 at 30°C.

Preparation of liposomes

Lipids dissolved in chloroform were combined in a glass tube to give the desired final composition. At this stage – if required – 2AS, 12AS or dansylPE were also included at concentrations of 1 mol% of total lipids (see below). The mixture was dried unter nitrogen and then hydrated by vortexing for 5 min with PBS to give a final lipid concentration of 4 mM. Small unilamellar vesicles (SUV) were prepared by sonification of the lipid solution for 10 min at ice temperature using a Branson sonifier (Danbury, USA).

Fluorescence measurements

Intrinsic fluorescence

All fluorescence spectra were recorded using an Aminco Bowman spectrometer series 2 (Rochester, USA). PDC-109 stock solutions (300 µM) were prepared in PBS. 5 µM PDC-109 was mixed with different amounts of lipid vesicles to give a molar lipid/protein ratio from 3 to 22, and the emission spectra were recorded with excitation at 280 nm (slit widths: 4 nm/4 nm).

Resonance energy transfer

Fluorescent phospholipid analogues labelled at the head group (dansylPE) or 2AS and 12AS with a fluorescent group at different positions of the fatty acid chain were introduced into liposomes of different composition (see above). The resonance energy transfer from the fluores-

cent amino acid residues of the protein to the dansyl or the anthroyloxy group, respectively, was measured. To this end 5 μM PDC-109 was mixed with different amounts of dansylPE-, 2AS- or 12AS-labelled liposomes. Fluorescence spectra were recorded using the following parameters: (i) dansylPE; λ_{ex} = 280 nm (for resonance energy transfer), λ_{ex} = 344 nm (for direct excitation of the dansyl group), and (ii) 2AS, 12AS; λ_{ex} = 280 nm (for resonance energy transfer), λ_{ex} = 364 nm (for direct excitation of the anthroyloxy group). In all cases the slit widths were 4 nm/4 nm.

Stopped-flow measurements

Kinetics of protein-membrane interaction were measured using a thermostatted stopped-flow device (RX 1000 Rapid Kinetics, Applied Photophysics, Surrey, UK) connected to an Aminco Bowman spectrometer series 2. The two chambers of the stopped-flow device were filled with 0.5 ml of the protein solution and 0.25 ml of the vesicle solution, respectively. The protein fluorescence was measured at 30 °C with λ_{ex} = 280 nm, λ_{em} = 336 nm (slits 4 nm/8 nm). The dead time of the stopped-flow apparatus is ≤ 10 msec. Ten single measurements under identical were accumulated in order to improve the signal to noise ratio.

ESR measurements

Spin-labelled lipids dissolved in chloroform/methanol (1:1, v/v) were transferred to a glass tube, dried under nitrogen and vortexed with the desired volume of PBS. ESR spectra in the absence and presence of PDC-109 were recorded at 25 °C using a Bruker ECS 106 spectrometer (Bruker, Karlsruhe, Germany). In another set of experiments 2 mM lipid vesicles were labelled with 0.05 mM SL-PC or SL-PE. After mixing those vesicles with 0.1 mM PDC-109 ESR spectra were recorded at 4 °C. Measuring parameters were as follows: modulation amplitude 2.5 G, power 20 mW, scan width 100 G, 16 times accumulated.

Results

Intrinsic fluorescence of PDC-109 in the presence of liposomes of various composition

The maxima of excitation and emission of the intrinsic fluorescence of PDC-109 in aqueous buffer (PBS) are at 280 nm and 342 nm, respectively (Fig. 1, inset), indicating that tryptophan residues are the major contributors to the fluorescence. After addition of SUV made of PC (i) the fluorescence intensity of PDC-109 measured at 333 nm was increased and (ii) the wavelength of the maximum was shifted to lower wavelengths (Figs. 1 and 2). Both alterations are typical for a more hydrophobic environment of the fluorescent amino acid residues. This suggests an interaction between the protein and the membranes. At a lipid/protein ratio of about10 the maximal fluorescence intensity was measured and the figure did not change further for higher lipid/protein ratios (Fig. 1). Likewise, the maximum blue shift of the fluorescence (333 nm) was obtained (Fig. 2). As a whole, these data suggest that 10 PC-lipids/PDC-109 molecule are required for maximal binding of PDC-109 to the vesicles.

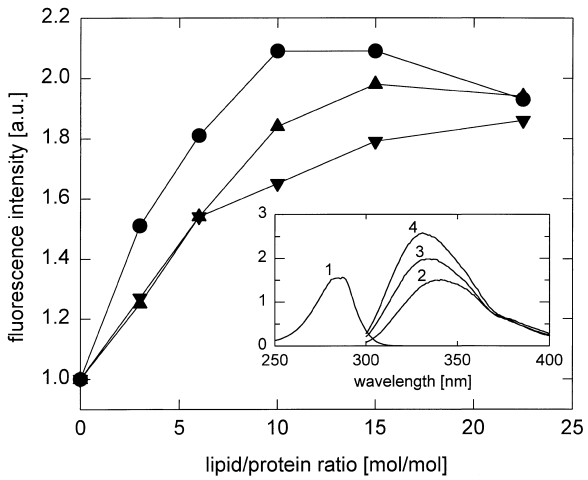

Fig. 1 Influence of the lipid composition of SUV on the intrinsic fluorescence of PDC-109. A 5 μM solution of PDC-109 in PBS (pH 7.4) was mixed with different amounts of lipid vesicles at 30°C and the fluorescence spectra were recorded (excitation wavelength was 280 nm). The fluorescence intensities measured at 333 nm normalized to the protein fluorescence in the absence of vesicles are given for PC SUV (●–●); PC/PE SUV (2:1) (▲–▲); PC/PS SUV (2:1) (▼–▼). The standard error of estimate was less than 6%. Inset: Fluorescence spectra (excitation at 280 nm) of 5 μM PDC-109 in PBS were recorded in the absence (*line 2*) and in the presence of 30 μM PC/PE SUV (2:1) (*line 3*) and PC SUV (*line 4*) at 30°C. *Line 1* shows the excitation spectrum of PDC-109 in PBS (emission at 340 nm)

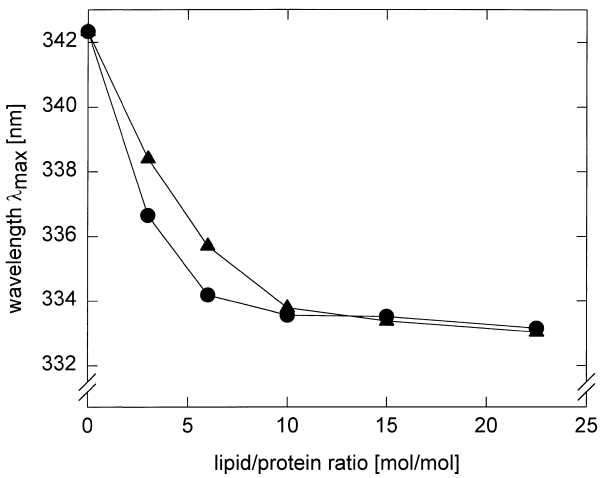

Fig. 2 Influence of the lipid composition of SUV on the intrinsic fluorescence of PDC-109. Solutions containing 5 μM PDC-109 in PBS (pH 7.4) were mixed with different amounts of lipid vesicles at 30 °C and the fluorescence spectra recorded (excitation wavelength was 280 nm). The wavelength λ_{max} of the fluorescence maximum is given for PC SUV (●–●); PC/PE SUV (2:1) (▲–▲)

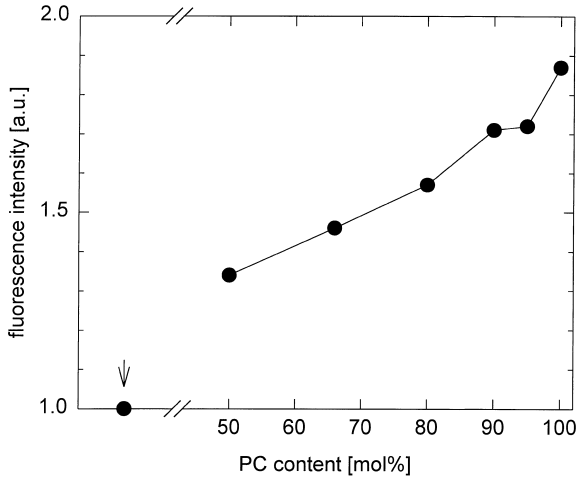

Fig. 3 Influence of PC content of SUV on the intrinsic fluorescence of PDC-109. 5 μM PDC-109 solutions in PBS (pH 7.4) were mixed with 30 μM PC/PE SUV made of different molar ratios of PC and PE at 30 °C and the fluorescence spectra were recorded (excitation wavelength was 280 nm). The fluorescence intensities measured at 333 nm are given. The *arrow* denotes the fluorescence intensity of PDC-109 in PBS which is normalized to 1

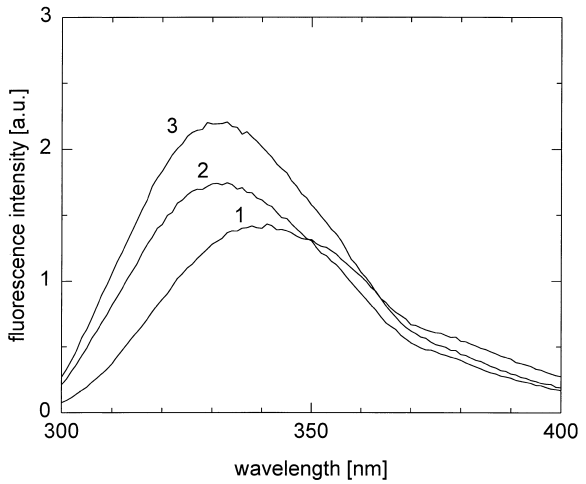

Fig. 4 Influence of lysoPC on the fluorescence spectrum of PDC-109. Fluorescence spectra of 5 μM PDC-109 in PBS (pH 7.4) were recorded in the absence (*line 1*) and in the presence of 30 μM (*line 2*) and of 112 μM (*line 3*) lysoPC at 30 °C. Excitation was at 280 nm

The intrinsic fluorescence intensity significantly depended on the composition of the liposomes (Figs. 1 and 2), and was largest when pure PC vesicles were used. Substituting part of PC for PE or PS, respectively, (PC : PE, PC : PS = 2 : 1 (mol : mol)) the increase of the intrinsic PDC-109 fluorescence intensity (Fig. 1) and the corresponding blue shift were lower with increasing lipid/protein ratios in comparison to pure PC vesicles (Fig. 2, PC/PS data not shown). As depicted in Fig. 3, the increase of the relative content of PC in PC/PE vesicles at a given lipid/protein molar ratio (6 : 1) was accompanied by a continuous enhancement of the intensity as well as by a blue shift of the protein fluorescence. The same behaviour was observed when replacing PC by SM (data not shown). The effect on the emission of PDC-109 fluorescence was more pronounced for the negatively charged substitute PS than for the neutral PE (Fig. 1), suggesting that electrostatic interaction may contribute to the binding of PDC-109 to the phospholipid vesicles. At PC/PE- and PC/PS-ratios above 15 and 22, respectively, neither enhancement nor further blue shift of PDC-109 fluorescence was detected. Since, under these conditions, the fluorescence parameters are similar to those of pure PC vesicles, we surmise that the interaction of PDC-109 with the lipid bilayer is identical to that of pure PC SUV.

The native protein structure seems to be required for the interaction of PDC-109 with membranes since denaturation of the protein by heating at 100 °C for 5 min abolished the change of the intrinsic protein fluorescence upon addition of liposomes (not shown). Interaction of PDC-109 with PC vesicles was not affected by the presence of 1 mM EDTA, implying that binding of PDC-109 does not require the presence of Ca^{2+}. Indeed, no significant effect of Ca^{2+} could be observed at concentrations up to 5 mM.

Interaction of lysophospholipids and spin-labelled phospholipids with PDC-109

To further characterize the lipid specificity involved in the interaction of PDC-109 with membranes we measured the intrinsic protein fluorescence in the presence of various lysolipids which are known to form micelles in aqueous suspension. Figure 4 shows emission spectra of PDC-109 in the presence of lysoPC. After addition of lysoPC to PDC-109 we found an increase of the fluorescence intensity as well as a blue shift of the maximum fluorescence. At a lipid/protein ratio of 22 the wavelength was shifted to 333 nm, which is comparable to the maximal shift measured with lipid bilayers. Addition of other lysolipids, such as lysoPS, did not have any effect on the intrinsic fluorescence of PDC-109, neither on the intensity nor on the wavelength of the maximum of fluorescence emission (not shown).

To further verify the specific interaction of PDC-109 by an independent approach we employed spin-labelled phospholipids to measure lipid-dependent parameters. Lipid analogues bearing a NO-moiety on the short fatty acid chain in the sn2-position are known to form micelles in aqueous buffer in a similar manner to lysolipids (Seigneuret et al. 1984). The ESR-spectra of those analogues dispersed in buffer are composed of two components: (i) an isotropic signal with three narrow lines arising from the free tumbling of the label monomers in buffer (Fig. 5 A, solid arrow), and (ii) a single broad component caused by strong spin-spin interaction of the label within the micelles (Fig. 5 A, dashed arrow). Spin-spin broadening is sensitive to the average distance between spin-labelled analogues. Thus, increasing the average distance between analogues reduces line broadening, separate peaks may then become visible and the typical ESR spectrum of the NO-moiety is

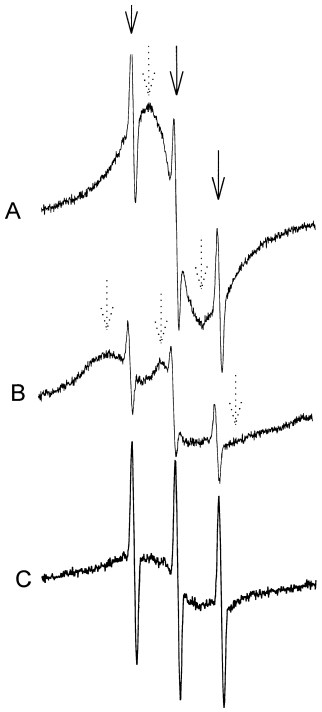

Fig. 5 Influence of PDC-109 on the ESR spectra of various spin-labelled lipids. The ESR spectra of 80 μM SL-PC (*A, B*) and SL-PE (*C*) were recorded in the absence (*A*) and in the presence (*B, C*) of 80 μM PDC-109 at room temperature as decribed in Materials and methods. The arrows denote the peaks of the free tumbling label monomers (*solid arrows*) and of the label arranged in micelles (*dotted arrows*), respectively

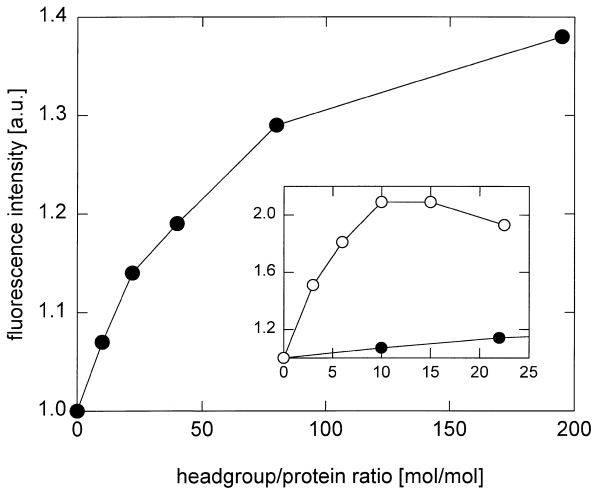

Fig. 6 Influence of phosphorylcholine chlorid (headgroup of PC) on the intrinsic fluorescence of PDC-109. 5 μM PDC-109 in PBS (pH 7.4) were mixed with different amounts of phosphorylcholine chloride at 30 °C and the fluorescence spectra recorded (excitation wavelength was 280 nm). In the inset data in presence of the PC headgroup (●–●) are compared with those in the presence of PC SUV (○–○) (data from Fig. 1). The fluorescence intensity at 333 nm normalized to the protein fluorescence in PBS is given

observed. The ESR spectrum obtained upon addition of PDC-109 to the spin-labelled analogue of PC (SL-PC) provided strong evidence for the interaction of PDC-109 with SL-PC micelles (Fig. 5B, dashed arrow). The lipid head group specificity of this interaction was obvious because separation of the broad component into those peaks was not observed for aqueous dispersions of SL-PE and SL-PS, respectively (Fig. 5C, only shown for SL-PE).

Interaction of PDC-109 with water soluble lipid head groups

To elucidate whether head group specificity of PDC-109 with lipid membranes is also manifested in the absence of the hydrophobic core of lipid membranes, we studied the influence of the water soluble head groups of phospholipids on the intrinsic protein fluorescence. Addition of phosphorylcholine to PDC-109 resulted in an increase of the intrinsic fluorescence measured at 333 nm (Fig. 6). In parallel, a continuous blue shift of the fluorescence spectrum with a maximum at 337 nm upon increasing the concentration of phosphorylcholine relative to that of PDC-109 (measured up to a ratio of 200 mol : mol) was observed (not shown). However, no change of the PDC-109 fluorescence was found in the presence of the water soluble head groups phosphorylethanolamine and phosphorylserine, respectively, even at a head group/protein ratio of 200 (mol : mol) (not shown).

Kinetics of PDC-109 binding to membranes

To measure the kinetics of the binding of PDC-109 to PC vesicles we employed a stopped-flow instrument in conjunction with a fluorescence spectrophotometer. The dead time of this system was ≤ 10 msec. Binding was followed by monitoring the increase of the intrinsic fluorescence of PDC-109. As shown in Fig. 7 binding was very rapid. 1 sec after mixing PDC-109 with lipid vesicles, the fluorescence had almost reached its final plateau. Nonlinear regression analysis suggests that the kinetics of fluorescence increase are at least biphasic (Fig. 7). The half-times deduced from a bi-exponential fit are 84 msec and 850 msec. The fast component comprises 65% of the total fluorescence increase. To identify the molecular origin of the two exponentials requires additional experiments and is beyond the scope of our study.

Interaction of PDC-109 with the polar membrane surface of liposomes

In order to characterize the binding of PDC-109 to the surface of liposomes the resonance energy transfer was measured from the fluorescent aromatic amino acid residues of the protein to a dansyl moiety covalently linked to the head group of a phospholipid (dansylPE). DansylPE was incorporated into PC SUV at a low concentration (1 mol%, see

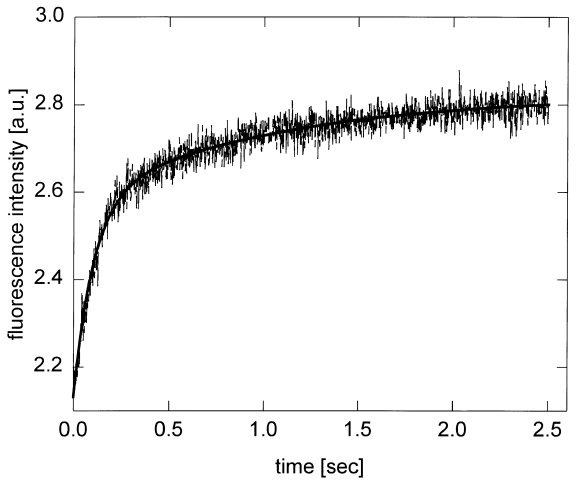

Fig. 7 Kinetics of the fluorescence increase of PDC-109 upon addition of PC SUV. 5 μM PDC-109 were mixed with 100 μM PC SUV in a stopped-flow device (see Materials and methods) and the intrinsic protein fluorescence at 333 nm was measured at 30 °C. Excitation wavelength was 280 nm. The mean of 10 accumulated scans is shown. A fit of the data by nonlinear regression analysis to a biexponential model is also shown

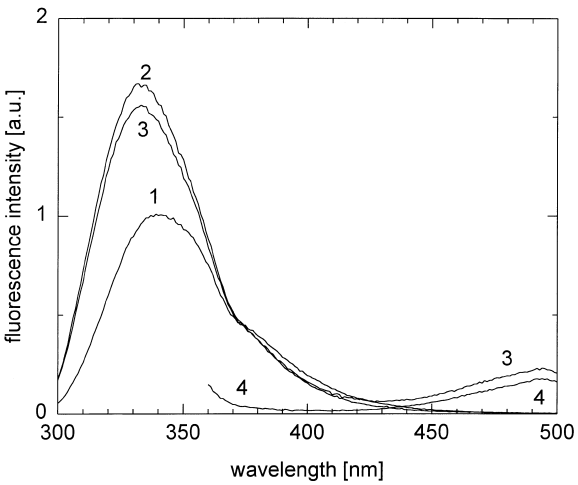

Fig. 8 Resonance energy transfer from the tryptophan groups of PDC-109 to dansyl-labelled PE (dansylPE). DansylPE (1 mol%) was incorporated into PC SUV and the vesicles were mixed with PDC-109 at 30 °C. The fluorescence spectra of 5 μM PDC-109 were recorded in PBS in the absence (*line 1*) (excitation = 280 nm), in the presence of 112 μM PC SUV (excitation = 280 nm) (*line 2*) and in the presence of PC/dansylPE SUV (resonance energy transfer: excitation = 280 nm, *line 3*; direct excitation of dansyl group = 338 nm, *line 4*)

Materials and methods) to perturb the interacting system as little as possible. Upon mixing the protein with labelled vesicles, PDC-109 fluorescence was measured by exciting the sample at the wavelength of tryptophan residues (280 nm). Figure 8 shows that (i) the protein fluorescence was quenched and that (ii) the fluorescence of the dansyl group was enhanced. These observations provide strong evidence for the occurrence of energy transfer between protein tryptophan groups and lipid dansyl moieties. We note a rather high degree of resonance energy transfer of dansylPE labelled liposomes when comparing the fluorescence spectrum of dansylPE by direct excitation (338 nm). This might argue for preferential incorporation of dansylPE with its bulky head group into the outer membrane leaflet of the liposomal membrane. One also has to consider that upon interaction of PDC-109 with liposomes the quantum yield may be affected by an altered microenvironment of the dansyl group.

Interaction of PDC-109 with the hydrophobic core of liposomes

The characteristics of the intrinsic fluorescence of PDC-109 upon binding to membranes (see above) are indicative of a hydrophobic environment of the fluorescent aromatic amino acids, most likely tryptophans. To investigate whether this is caused by incorporation of PDC-109 into the hydrophobic part of the membrane, PC-liposomes labelled with an anthroyloxy fatty acid bearing the fluorescent anthroyloxy group at different positions of the fatty acid chain, 2AS and 12AS (see Materials and methods) were prepared. The fluorescent group is located about 1 Å (2AS) and 9 Å (12AS) from the membrane surface (Clague et al. 1991). Addition of PDC-109 to labelled liposomes caused resonance energy transfer from the protein tryptophan group(s) (excitation at 280 nm) to the anthroyloxy group of both fatty acids as indicated by (i) quenching of the protein fluorescence in comparison to non-labelled vesicles and (ii) an increase in the anthroyloxy fluorescence (Fig. 9). The degree of resonance energy transfer was slightly higher for 2AS-labelled SUV than for 12AS-labelled SUV (compare Fig. 9 A and B). The higher intensity of the anthroyloxy fluorescence (upon excitation at 364 nm) in the case of 12AS-labelled SUV might be due to the deeper localization of this fluorophore in the lipid bilayer and, therefore, more hydrophobic environment (compare spectra 4 of Fig. 9 A and B).

Binding of PDC-109 affects membrane fluidity of liposomes

The spin-labelled phospholipid analogue of PC (SL-PC) was incorporated at 2.5 mol% into the membrane of PC and PC/PE SUV after vesicle preparation. This ensures that all labelled lipids are oriented on the outer leaflet since the redistribution of those analogues across the bilayer is a very slow process with a half-time of several hours. Using this approach, we could only monitor alterations of the outer leaflet occurring upon binding of PDC-109. The ESR spectra of labelled vesicles were recorded in the absence and presence of PDC-109 at 4 °C. This low temperature was chosen in order to allow deconvolution of the components from the spectra which are due to protein-lipid interaction. A rapid exchange of labelled lipids between the bulk phase and the protein neighbourhood, favoured by increasing the temperature, would significantly hamper such deconvolu-

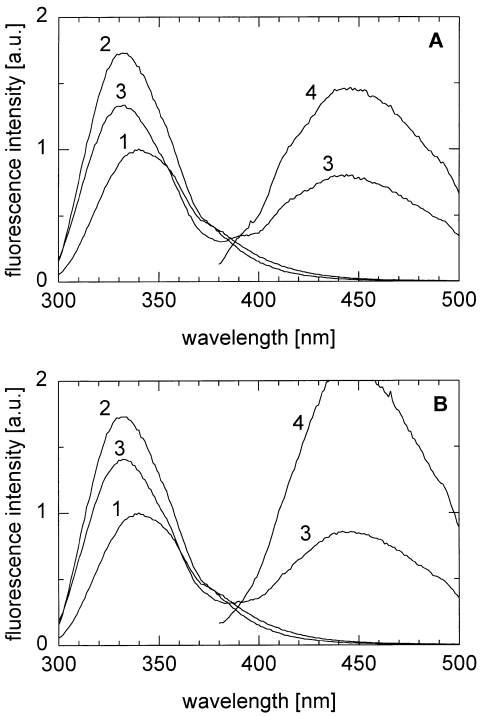

Fig. 9 A, B Resonance energy transfer from the tryptophan groups of PDC-109 to 2- and 12-(9-anthroyloxy)stearic acid (2AS, 12AS). 2AS (**A**) and 12AS (**B**) were incorporated at 1 mol% into PC SUV. Vesicles were mixed with 5 μM PDC-109 and the fluorescence spectra of the protein were recorded at 30 °C in the absence (*line 1*), in the presence of 30 μM 2AS or 12AS labelled SUV (resonance energy transfer: excitation = 280 nm, *line 3*; direct excitation = 364 nm, *line 4*) and in the presence of 30 μM non-labelled SUV (*line 2*)

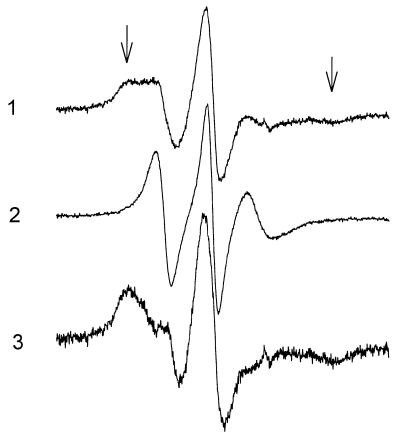

Fig. 10 Influence of PDC-109 on the ESR spectra of spin-labelled PC incorporated into SUV membranes. 2 mM PC SUV were labelled with 0.05 mM SL-PC and the ESR spectra were recorded at 4 °C in the absence (*spectrum 2*) and in the presence (*spectrum 1*) of 0.1 mM PDC-109. In the latter case a strongly immobilized component was seen (see *arrows*) which was extracted by subtraction of spectrum 2 from spectrum 1 (*spectrum 3*)

tion. In Fig. 10 (spectrum 2) a typical membrane spectrum of SL-PC in PC vesicles at 4 °C in the absence of PDC-109 is shown. From the shape of the spectrum it can be deduced that no clustering of the labelled molecules in the liposomal membrane occurs. Upon addition of PDC-109, a second, strongly immobilized component in the ESR spectra was observed (indicated by the two outer wings in Fig. 10, spectrum 1), indicating a strong interaction of the protein with the lipid phase leading to a decrease of the membrane fluidity. Measurements were performed at the rather high lipid/protein ratio of 20 : 1 to ensure almost complete binding of the protein to membranes (see above). The double integral of the spectra, which is a direct measure of spin label concentration, is similar in the absence and in the presence of the protein. This enabled us to quantify the immobilized spectrum. By subtraction of the ESR spectrum in the absence of PDC-109 from that in the presence of the protein we estimated that about 76% of the labelled molecules contribute to the immobilized component (SL-PC in PC SUV, Fig. 10, spectrum 3). Taking into account that about 70% of non-labelled lipids (Thomas and Poznansky 1989) and all SL-PC are localized in the outer leaflet of SUV, we estimated that one PDC-109 molecule interacts with about 11 lipid molecules. This figure is consistent with the results of the fluorescence experiments (see above). Furthermore, we found a similar value when the corresponding spectra of SL-PC in PC/PE SUV (PC : PE = 2 : 1) at the lipid/protein molar ratio of 20 : 1 were measured (data not shown).

Discussion

In the present study we have investigated the interaction of PDC-109 with phospholipid vesicles directly and on a quantitative level. The major conclusions of our results are (i) PDC-109 interacts specifically with phosphorylcholine lipids incorporated into small unilamellar vesicles, confirming previous studies of Desnoyers and Manjunath (1992; 1993); (ii) the binding of PDC-109 to membranes is a very rapid process with half times of less than one second; (iii) PDC-109 interacts not only with the solvent-exposed choline group but also with the apolar part of the lipid bilayer; (iv) binding of PDC-109 to the vesicle membrane results in a rigidification of the lipid phase. These conclusions are discussed in more detail below, and are interpreted within the framework of present knowledge and hypotheses on the function of PDC-109 in bovine sperm capacitation.

The results of the different approaches used converge to indicate that the phospholipid headgroup, the phosphorylcholine moiety, is the main determinant of the specific binding of PDC-109 to the membrane surface. Stepwise increase of the lipid/protein ratio caused a steep increase in the intrinsic fluorescence of PDC-109 in the presence of pure PC vesicles (Figs. 1–4). Above a lipid/protein ratio of 10 : 1 the fluorescence did not change further, indicating that binding sites for PDC-109 on the vesicle surface were saturated. An almost identical estimate (11 lipids/PDC-109 molecule) was obtained by an independent approach employing ESR-spectroscopy in con-

junction with spin-labelled PC (Fig. 10). Decreasing the PC density by incorporation of PS or PE in the vesicles impaired binding of PDC-109 to membranes as deduced from the reduced increase of protein fluorescence at low lipid/protein ratios. In agreement with this, saturation of the fluorescence increase of PDC-109 by interaction with mixed PC/(PE or PS) vesicles was achieved at significantly higher lipid/protein ratios in comparison with pure PC vesicles. In the case of PC/PE vesicles the fluorescence plateau was reached at a lipid/protein ratio of about 15 (Fig. 1). At this ratio the PC concentration is similar to that at which the maximum fluorescence of PDC-109 was attained in the case of pure PC vesicles (Fig. 2). Using equilibrium dialysis, Desnoyers and Manjunath (1993) have shown that in solution a PDC-109 molecule binds on average 1.8 choline molecules with an overall binding constant of 0.95 mM. Our data suggest that the density of phosphorylcholine groups contribute to the efficiency of PDC-109 binding to the membrane surface.

For PC-liposomes containing negatively charged PS, the lipid/protein ratio with which maximum fluorescence was observed was above 20 : 1. This suggests that electrostatic repulsion may significantly reduce the binding of PDC-109 to the lipid membrane. This effect can be rationalized because PDC-109 is an acidic protein with a pI in the range 5–5.5 (Desnoyers et al. 1994). Negatively charged residues (10 aspartic acids and 7 glutamic acids per PDC-109 molecule) correspond to 16% of the total amino acids of the protein (Esch et al. 1983). Notably, most of the negative charges are concentrated within the N-terminal 23 amino acids of PDC-109. This polypeptide stretch contains 4 Asp and 6 Glu residues (Esch et al. 1983), suggesting the existence of a discrete and highly negatively charged domain within the PDC-109 molecule.

The change of the intrinsic fluorescence intensity of PDC-109 upon interaction with phosphorylcholine-containing lipids may be caused either by interaction of the lipid head group with protein sequences close to aromatic amino acid residues and/or by conformational change(s) of the PDC-109 molecule. The observation that soluble phosphorylcholine could only partly mimic the interaction of the protein with PC-membranes, and the failure of this soluble ligand to induce such a pronounced effect on the intrinsic fluorescence intensity of PDC-109 as that caused by PC vesicles (Fig. 6), strongly indicate that PDC-109 did not interact solely with the head group of PC but also with the hydrophobic part. Furthermore, the decreased line broadening of the ESR spectrum of spin-labelled PC analogues in micelles can be explained by assuming an interaction of PDC-109 with SL-PC (Fig. 5), presumably causing an enhanced average distance between the NO moieties of the labelled analogues. This would imply that PDC-109 does not associate only with the surface of the SL-PC micelles, in which case we would not expect an increase of the average distance between analogues, but may interact with the hydrophobic part of SL-PC. This is very likely since the presence of a solvent exposed hydrophobic region has been indicated by the observation that PDC-109 has affinity for the neopentyl groups of alkyl-Sepharose (Bányai et al. 1990) and the diethylaminoethyl groups of DEAE-Sephadex (Desnoyers and Manjunath 1993, Calvete et al. 1996b) even at high ionic strength (1 M NaCl).

Resonance energy transfer between aromatic amino acids (donor) and the fluorescent anthroyloxy-group (acceptor) attached to a fatty acid either close to the carboxy group (head group region) or at a position close to the midplane of the bilayer further supported the hypothesis of the intercalation of the protein into the membrane bilayer. Energy transfer between the protein and the anthroyloxy-group localized in the head group region and in the hydrophobic part of the bilayer may indicate that only a part of the protein will interact with the hydrophobic core, leaving the rest of the molecule oriented towards the polar head group.

It has recently been shown that phospholipids are asymmetrically distributed between the exoplasmic and the cytoplasmic leaflet of the plasma membrane of ram and bull sperm cells (Müller et al. 1994, Nolan et al. 1995, Müller et al. 1997a). It was found that PC is preferentially localized in the outer membrane leaflet while the aminophospholipids PS and PE are mainly oriented to the inner leaflet. PS and PE are efficiently removed from the exoplasmic leaflet by an ATP-dependent transport to the cytoplasmic layer. This transport, which is specific for aminophospholipids, is characterized by a half-time of about 3 min. Similar transbilayer dynamics and distribution of phospholipids exist in epididymal bovine spermatozoa (Müller et al. 1997b). Hence, PC-containing lipids are properly located to act as anchoring sites for PDC-109, and other PC-binding molecules (i.e. BSP-A3 and BSP-30K). Interestingly, although phosphorylcholine-containing lipids represent over 60% of the total lipids of bull spermatozoa (Watson 1981), PDC-109 preferentially coats the postacrosomal and midpiece regions of bovine spermatozoa (Manjunath et al. 1994). A nonhomogeneous distribution of lipids, as shown for glycolipids (Gadella et al. 1995), creating phosphorylcholine-rich domains at these sperm locations could potentially explain the differential distribution of sperm-bound PDC-109 molecules.

Our results show that the interaction of PDC-109 with PC-lipids has significant consequences for the physical properties of the membrane. Thus, using spin-labelled PC we found a significant rigidification of the lipid upon addition of PDC-109 to PC vesicles (Fig. 10). Further studies are needed to characterize in detail the membrane alterations caused by PDC-109. Perturbation of the membrane physical characteristics (i.e., fluidity) have been shown to be relevant for membrane functions such as transport and the activity of membrane associated enzymes. In particular, membrane remodelling has been invoked as the molecular basis of sperm capacitation. Manjunath and coworkers (1994) have put forward a model whereby BSP proteins (PDC-109, BSP-A3, and BSP-30K), upon ejaculation, coat the spermatozoal surface by specific interaction with PC. This may decapacitate spermatozoa and prevent premature acrosome reactions. BSP proteins (and presumably PDC-109 which accounts for over 80% of the total proteins of bovine seminal plasma) (Calvete et al. 1994) may act as docking molecules for apolipopro-

tein (apo) A1/high-density lipoprotein (HDL) complexes. Release of these macromolecular assemblies during sperm residence in the female's genital tract (perhaps triggered by interaction with heparin-like glycosaminoglycans) may sequester cholesterol and PC-containing lipids. This loss of lipids may change membrane characteristics and promote capacitation. Our results showing that binding of PDC-109 to lipid vesicles alters the fluidity of the lipid bilayer would support the proposed decapacitating activity of PDC-109.

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