The Lipid Composition Modulates the Influence of the Bovine Seminal Plasma Protein PDC-109 on Membrane Stability†

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ABSTRACT: The bovine seminal plasma protein PDC-109 exerts an essential influence on the sperm cell plasma membrane during capacitation. However, by any mechanism, it has to be ensured that this function of the protein on sperm cells is not initiated too early, that is, upon ejaculation when PDC-109 and sperm cells come into first contact, but rather at later stages of sperm genesis in the female genital tract. To answer the question of whether changes of the bovine sperm lipid composition can modulate the effect of PDC-109 on sperm membranes, we have investigated the influence of PDC-109 on the integrity of (i) differently composed lipid vesicles and of (ii) membranes from human red blood cells and bovine spermatozoa. PDC-109 most effectively disturbed lipid membranes composed of choline-containing phospholipids and in the absence of cholesterol. The impact of the protein on lipid vesicles was attenuated in the presence of cholesterol or of noncholine-containing phospholipids, such as phosphatidylethanolamine or phosphatidylserine. An extraction of cholesterol from lipid or biological membranes using methyl-β-cyclodextrin caused an increased membrane perturbation by PDC-109. Our results argue for a oppositional effect of PDC-109 during sperm cell genesis. We hypothesize that the lipid composition of ejaculated bull sperm cells allows a binding of PDC-109 without leading to an impairment of the plasma membrane. At later stages of sperm cell genesis upon release of cholesterol from sperm membranes, PDC-109 triggers a destabilization of the cells.

The seminal plasma of mammalians contains a number of proteins that are involved in the modulation of sperm cell genesis (*1*). Among these, the Fn type II protein family has gained special attention (*2*). Fn type II proteins are characterized by a conserved gelatin-binding domain, which was originally described in fibronectin (*3*−*5*). Proteins of this family have been found in the male genital tract of various mammalian species such as cattle (BSP) (*6*), horse (HSP or SP) (*7*−*9*), goat (*10*), pig (pB1) (*11*), bison (*12*), dog, and human (*13*). The Fn type II domain in these proteins is tandemly two-fold (*6*−*8*, *11*) or four-fold (*13*, *14*) arranged, additionally carrying N-terminal polypeptide extensions of variable lengths. Recently, it was found that those proteins belong to separate families of sperm-coating proteins by performing a phylogenetic analysis (*15*). Experimental data from the bovine (BSP) proteins indicate an influence of Fn type II proteins during the capacitation process of sperm cells (*16*, *17*). However, on the basis of structural differences between Fn type II proteins and/or differences in the membrane composition of sperm cells from various species, other functions of these proteins have been surmised that imply species-specific effects of the proteins in the course of sperm cell genesis (*14*, *18*).

The most prominent representative of the Fn type II protein family in mammalian seminal plasma is the bovine PDC-109 (also named BSP-A1/A2), which contains two glycoforms of the same polypeptide chain (*19*, *20*). PDC-109 is secreted by seminal vesicles and binds upon ejaculation to the plasma membrane of sperm cells (*21*, *22*). The binding site for the protein has been identified as phospholipids carrying a choline headgroup, that is, phosphatidylcholine (PC)[1] and sphingomyelin (SM) (*23*−*25*). Membrane binding takes place at a stoichiometry of about 10 lipid (PC) molecules per one protein molecule (*24*−*26*). Recently, the crystal structure of PDC-109 with a bound phosphorylcholine moiety was resolved, showing that the protein binds choline moieties by cation π-interaction (*27*). A physiological role of PDC-109 was concluded from the fact that it enhances the capacitation of epididymal sperm cells in the presence of heparin and HDL (*16*, *17*).

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[1] Abbreviations: C6-NBD-PC, 1-palmitoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphocholine; HBS, Hepes-buffered salt solution; LUV, large unilamellar vesicle(s); MCD, methyl-β-cyclodextrin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, propidium iodide; P/L, molar ratio protein to lipid; SM, sphingomyelin; SUV, small unilamellar vesicle(s).

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Upon interaction with membranes, PDC-109 was shown to affect membrane structure. First, the binding of PDC-109 to lipid vesicles or to sperm cells causes a reduction of the mobility of membrane lipids, which has been assumed to prevent the cells from a premature triggering of capacitation (*18*, *24*, *25*, *28*−*30*). Second, PDC-109 is able to extract lipids, namely, PC and cholesterol, in a selective manner, from sperm cells affecting their plasma membrane composition (*31*−*34*). The modulation of the plasma membrane composition, in particular with respect to cholesterol content, seems to be a key process during sperm capacitation (*35*−*38*). This is underlined by the fact that capacitation of sperm cells could be induced solely by incubating cells with methyl-β-cyclodextrin (MCD) (*39*−*42*), which is known to extract cholesterol from membranes (*43*, *44*). Third, it has been shown that PDC-109 impairs membrane integrity (*25*, *26*, *28*, *45*). Within these studies, which were solely performed on lipid vesicles, for example, an enhanced leakage of aqueous content markers in the presence of PDC-109 was observed (*26*).

The results on the interaction of PDC-109 with membranes reveal opposite effects of the protein on membrane structure and stability. In search of the reason(s) for these contradictory effects, it can be surmised that the lipid composition modulates the influence of PDC-109 on membrane stability. Therefore, in the present study, we have characterized the influence of PDC-109 on the integrity of liposomal and biological membranes. We were interested in whether the lipid composition of membranes might modulate the impact of the protein on membrane integrity. Because cholesterol plays a specific role during sperm cell capacitation, we investigated whether changes in the cholesterol content of membranes may influence protein−membrane interaction. We found that PDC-109 most effectively disturbed membranes composed of choline-containing phospholipids in the absence of cholesterol. The effect of PDC-109 was diminished in membranes containing either other phospholipids [phosphatidylethanolamine (PE)] or cholesterol. An extraction of cholesterol from membranes by MCD in the presence of PDC-109 caused an increase of membrane perturbation. Our results argue for a dual effect of seminal Fn type II proteins during sperm cell genesis.

EXPERIMENTAL PROCEDURES

Chemicals. NBD-labeled PC, 1-palmitoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphocholine (C6-NBD-PC), was obtained from Avanti Polar Lipids (Birmingham, AL). Calcein was from Fluka Feinchemikalien GmbH (Neu-Ulm, Germany). All other chemicals were from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Hepes-buffered salt solution (HBS) contained 5mM Hepes and 145 mM NaCl and was set to pH 7.4.

PDC-109 was purified from the seminal plasma of reproductively active Holstein bulls by a combination of affinity chromatography on heparin-Sepharose and DEAE-Sephadex chromatography as described (*46*). The protein purity was verified by sodium dodecyl sulfate−polyacrylamide gel electrophoresis, reverse phase high-performance liquid chromatography analysis, and mass spectrometric analysis. Purified PDC-109 consists of a roughly equimolar mixture of glycosylated (13433 Da) and nonglycosylated (12788 Da) species (*20*). For the experiments, a 1 mM stock solution of the protein was prepared in HBS. The protein concentration was determined using the Roti-Nanoquant (Carl-Roth GmbH + Co., Karlsruhe, Germany) protein assay.

Lipid Extraction from Bull Sperm Cells. Bull semen was obtained from different fertile animals of a breeding station (Rinderbesamung Berlin-Brandenburg GmbH, Besamungsbullenstation Schmergow, Germany). Aliquots of 1 mL of each ejaculate were centrifuged at room temperature (12000*g*, 2 min), and the seminal plasma was removed by pipet. The sperm cell sediment was carefully resuspended with 1 mL of 0.9% NaCl, and the supernatant was discarded after centrifugation (12000*g*, 2 min). The cell pellets obtained from different animals were pooled, and after a second washing step, the cell concentration was adjusted with 0.9% NaCl to about 4×10^8 spermatozoa/mL. Pellets were frozen at −20 °C until use for lipid extraction.

Lipids were extracted from sperm cells according to the method of Bligh and Dyer (*47*). Briefly, cells were suspended in 0.8 mL of bidistilled water. Afterward, 3 mL of a chloroform/methanol mixture (1:2 v/v) was added, and the sample was vigorously vortexed for 1 min at room temperature. One milliliter of chloroform was added, and the sample was again vortexed. After the addition of 1 mL of 40 mM acetic acid, vortexing, and centrifugation (5 min, 1000*g*), the organic layer was removed. The aqueous phase was again mixed with 2 mL of chloroform, vortexed, and centrifuged. The organic layer was removed, combined with the other one, and used for further experiments.

Preparation of Lipid Vesicles. Aliquots of lipids in organic solution were transferred to a glass tube and dried under nitrogen. The lipids were resuspended in a small volume of ethanol, and HBS was added to give a final lipid concentration of 1−4 mM [the final ethanol concentration was below 1% (v/v)]. To prepare large unilamellar vesicles (LUV), five freeze−thaw cycles were performed followed by extrusion of the lipid solution 10 times at 40 °C through two 0.1 μm polycarbonate filters (miniextruder from Avanti Polar Lipids, Alabaster, AL; filters from Costar, Nucleopore GmbH, Tübingen, Germany).

In the case of liposomes made from lipids of bull spermatozoa, the preparation of LUV was accompanied with a high loss of lipids in the filter. Therefore, small unilamellar vesicles (SUV) were prepared by sonification of the lipid extracts obtained from bull sperm cells (Branson Sonifier 250, Schwäbisch Gmünd, Germany, intensity 2, cycle 80%).

Lipid Determination. The phospholipid and cholesterol content of LUV was routinely determined after running calcein-loaded vesicles on PD 10 columns (see below). For this purpose, lipids were first extracted into chloroform (*47*), which was then evaporated under a stream of nitrogen. The phospholipid content was determined by measurement of phosphorus as described (*48*). The cholesterol content was determined using a colorimetric kit (R-Biopharm GmbH, Darmstadt, Germany).

Measurement of Calcein Leakage. LUVs or SUVs (in the case of bull sperm lipids) were prepared in HBS containing calcein at a self-quenching concentration (70 mM), which results in a low fluorescence intensity. Calcein-filled vesicles were separated from bulk calcein using a PD-10 column (Sephadex) at room temperature and HBS as elution buffer. Leakage was measured by monitoring fluorescence de-

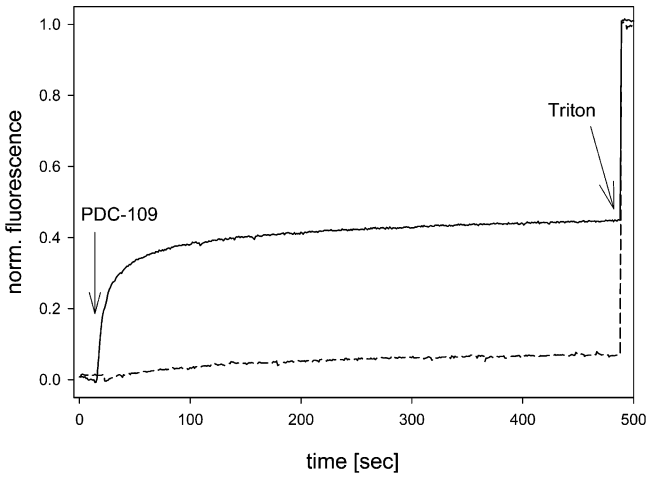

FIGURE 1: PDC-109-induced leakage of calcein from LUV. Calcein leakage from LUV composed of PC (solid curve) or PC containing 30 mol % cholesterol (dashed curve) was measured at 37 °C by following the time-dependent fluorescence increase. Leakage was induced by adding 5 μM PDC-109 to the LUV, giving a final protein to lipid ratio of 0.24 and 0.36 for PC- and PC/cholesterol-LUV, respectively. After about 480 s, Triton X-100 was added [final concentration 0.5% (w/v)] to obtain complete leakage of calcein, which was set to 100%.

quenching of calcein upon addition of PDC-109. For that, the fluorescence of vesicles in HBS was monitored at 515 nm (λ_{ex} = 490 nm; slit width for excitation and emission, each 4 nm) using an Aminco Bowman Series 2 spectrofluorometer (SLM-AMINCO, Rochester, NY). Because of the separation of vesicles on the PD-10 column, the final lipid concentration varied between 10 and 20 μM, resulting in different molar ratios of protein to lipid (P/L) among experiments. All measurements were performed at 37 °C while continuously stirring the solution. The maximal leakage was determined by the addition of 1% Triton X-100. The amount of leakage (L) was determined as

$$L = \frac{F_t - F_0}{F_{max} - F_0}$$

where F_0 and F_{max} refer to the initial fluorescence intensity of calcein-filled vesicles before the addition of PDC-109 and the fluorescence intensity after the addition of Triton X-100, respectively (see Figure 1). F_t denotes the amount of fluorescence reached about 400 s after the addition of PDC-109. Typically, a fluorescence plateau was reached within this period (Figure 1). Fluorescence intensities were corrected for dilution due to the addition of PDC-109 and Triton X-100.

To simulate a release of cholesterol, LUVs containing cholesterol were incubated with MCD, which extracts cholesterol from the membranes (*43*, *44*, *49*, *50*). If not stated otherwise, MCD was added from a 200 mM stock solution in HBS to a final concentration of 10 mM during the fluorescence measurement before the addition of PDC-109.

Reduction of NBD Fluorescence by Dithionite. LUV preparation was done in the presence of 1 mol % of C6-NBD-PC obtaining symmetrically labeled vesicles, that is, the fluorescent lipids were equally distributed between both membrane leaflets. Labeled LUVs were suspended in a cuvette (final lipid concentration, 25 μM), and the fluorescence of the NBD group was monitored continuously at 535 nm (λ_{ex} = 470 nm, slit width for excitation and emission, each 4 nm) at 25 °C. Different amounts of PDC-109 were added from a stock solution resulting in a fluorescence decrease. This decrease reflects an extraction of C6-NBD-PC from the membrane onto the protein since the fluorescence quantum yield of analogues bound to PDC-109 is lower as compared to those localized in the membrane (*34*). After 300 s, sodium dithionite was added from a freshly prepared 1 M stock solution in 100 mM Tris (pH 10) to give a final concentration of 25 mM. Dithionite rapidly quenches the fluorescence of analogues in the outer membrane leaflet by chemical reaction with their NBD groups (*51*). In the absence of PDC-109, dithionite permeates slowly across lipid membranes. Therefore, a plateau that is observed after the rapid fluorescence decrease reflects the fluorescence of analogues in the inner membrane leaflet, which are not accessible to dithionite (*51*). After the fluorescence intensity was reduced by dithionite to a plateau value (about 300 s), Triton X-100 was added to a final concentration of 0.5% (w/v), enabling access of the remaining analogues on the inner side of the membrane to dithionite, which resulted in a complete loss of fluorescence. The amount of analogues not accessible to dithionite (PC_n) was determined according to

$$PC_n = \frac{F_p - F_b}{F_i - F_b}$$

with F_p being the fluorescence of the plateau after dithionite reduction, F_b the background fluorescence after the addition of Triton X-100, and F_i the initial fluorescence intensity before addition of PDC-109 and dithionite.

In the presence of PDC-109, fluorescence intensities decreased to lower plateau values upon addition of dithionite; that is, the analogues on the inner leaflet became accessible to dithionite due to an increased permeation of dithionite (kinetics not shown). The extent of PDC-109-induced dithionite permeation was calculated from the kinetics according to

$$\text{permeation} = \frac{PC_{n/wo} - PC_{n/PDC}}{PC_{n/wo}}$$

with $PC_{n/wo}$ and $PC_{n/PDC}$ representing the fraction of dithionite-inaccessible analogues in the absence and in the presence of PDC-109, respectively.

Measurement of Intrinsic Fluorescence of PDC-109. PDC-109 was diluted from a stock solution with HBS to a concentration of 2.5 μM in a fluorescence cuvette. Fluorescence spectra of the tryptophan residues were recorded using an Aminco Bowman Series 2 spectrofluorometer in the range of 300−400 nm (λ_{ex} = 280 nm; slit width for excitation and emission, each 4 nm) at 37 °C .

PDC-109-Induced Hemolysis of Erythrocytes. Citrate-stabilized blood was obtained from the blood bank in Berlin-Lichtenberg, Germany. After removal of the buffy coat, erythrocytes were washed twice in HBS at 4 °C and resuspended to a hematocrit of 10%. PDC-109 was added at various concentrations to aliquots of this suspension and incubated for 10 min at 37 °C. Subsequently, erythrocytes were pelleted by centrifugation. The degree of hemolysis was

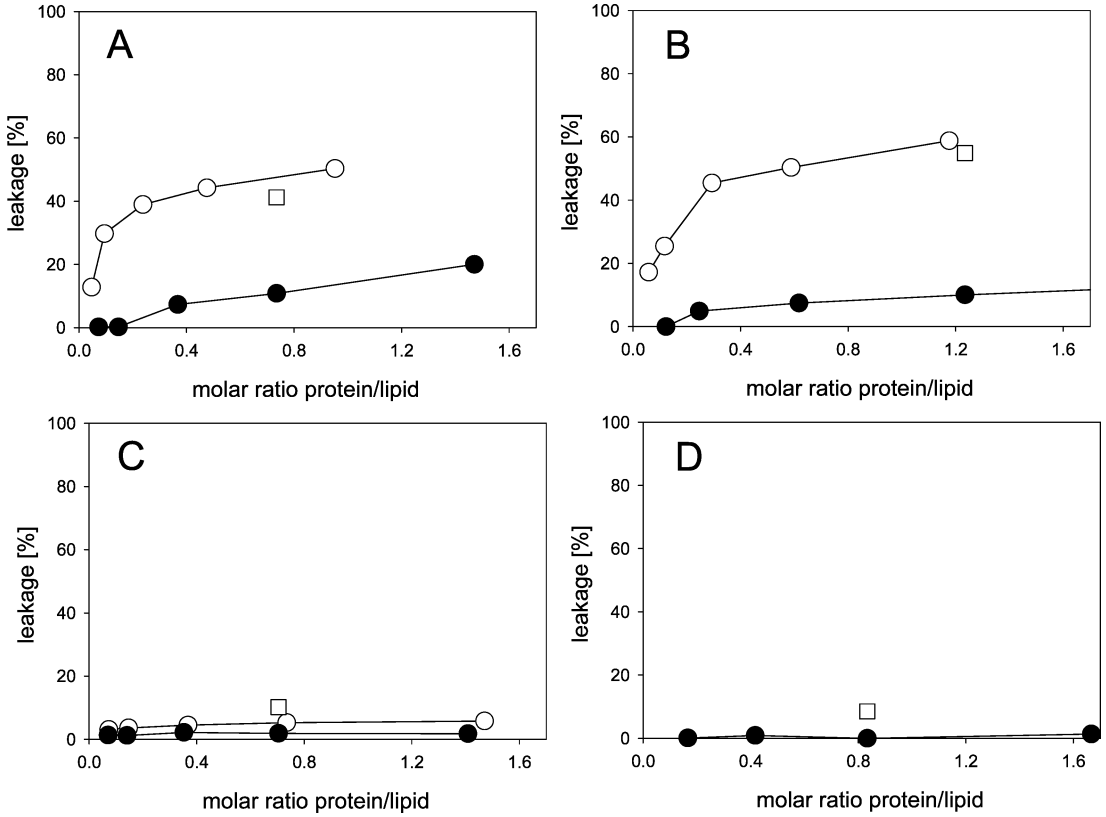

FIGURE 2: PDC-109-induced leakage of calcein from lipid vesicles of various compositions. The leakage of calcein was measured from vesicles after incubation with various amounts of PDC-109 at 37 °C as described in the Experimental Procedures. LUV (or SUV in the case of D) contained PC (A), PC/SM (5:2) (B), PC/PE (1:1) (C), and lipids extracted from bull sperm cells (D). In A−C, vesicles contained solely the respective phospholipids (open circles) or additionally 30 mol % cholesterol (filled circles). In some experiments, cholesterol-containing vesicles were preincubated with 10 mM MCD for 10 min before the addition of PDC-109 (open squares). Each figure shows the data of one representative experiment.

followed by measuring the absorption of the supernatant at 540 nm. The value of 100% hemolysis was obtained by the addition of Triton X-100 (final concentration 1%). To decrease the cholesterol content in erythrocyte membranes, the cells were preincubated with 10 mM MCD at 37 °C before the addition of PDC-109.

Preparation of Bull Epididymal Sperm Cells. Bovine testes were obtained from the local slaughterhouse. The epididymes from two animals were dissected according to ref *52*. Spermatozoa from the cauda epididymis were obtained by fine mincing of epididymal tissue in a petri dish with glucose-supplemented Hank's solution (without calcium and magnesium) and subsequent filtration through paper tissues. Spermatozoa were then centrifuged (600*g*, 5 min), and the pellet was resuspended in 20 volumes of HBS. After a second centrifugation, cells were pooled and resuspended in HBS again to about 3×10^8 cells/mL. This corresponds to a plasma membrane lipid concentration of about 0.5 mM, assuming a lipid content of 1.7 μmol for the plasma membrane of 10^9 sperm cells (*53*).

Measurement of Sperm Cell Integrity. To discriminate between intact and impaired spermatozoa from the cauda epididymes, we used the DNA stain propidium iodide (PI), which selectively permeates the plasma membrane of dead cells. The sperm cell suspension (100 μL) was mixed with 1 μL of PI (stock 1.5 mM) and incubated for 5 min at 38.5 °C in the dark. Subsequently, sperm cells were incubated (i) without, (ii) with PDC-109 (giving a P/L = 1:10), (iii) with 5 mM MCD, or (iv) with PDC-109 (P/L = 10) and MCD (5 mM). After 10 min of coincubation at 38.5 °C in the dark, aliquots were analyzed by flow cytometry. For that, 10 μL of labeled cell suspension was gently diluted with 2 mL of prewarmed buffer in a measuring cuvette (about 10^6 cells/mL) and measured in a flow cytometer (Partec GmbH, Münster, Germany) equipped with a 400 mW argon laser (ex 488 nm), and a 620 nm long-pass filter for PI. The system was triggered on the forward light scatter, and 10000 cells per sample were characterized for their fluorescence.

RESULTS

PDC-109-Induced Leakage of Calcein. To assess a PDC-109-induced perturbation of membrane integrity, we determined the leakage of calcein from LUV of different lipid composition. The addition of PDC-109 to pure PC-LUV induced a remarkable release of the dye with kinetics following an exponential rise to a maximum (Figure 1). The degree of the leakage increased with rising protein concentration (Figure 2A). Already at low protein/lipid ratios (P/L < 0.2), about 30% of calcein was released from the vesicles, whereas at larger protein concentration (P/L > 0.2) this value increased to more than 40%. The leakage of calcein from PC-LUV could be inhibited by preincubating PDC-109 with phosphorylcholine but not with phosphorylethanolamine (data not shown), underlining the head group specificity of the protein. Incorporation of cholesterol (30 mol %) significantly reduced PDC-109-induced leakage; at P/L below 0.2,

no leakage was observed, and at a P/L of around 1, less than 20% of the dye was released.

Because the plasma membrane of bull spermatozoa contains a variety of lipids including PC and cholesterol, we addressed how the presence of other phospholipids in the vesicle membrane modifies the PDC-109-mediated release of calcein. For PC/SM-LUV, the release of the dye upon additon of PDC-109 was similar to that found for PC-LUV (Figure 2B). When cholesterol (30 mol %) was present in PC/SM-LUV, the protein induced much smaller leakage (Figure 2B). In contrast, PDC-109 did not perturb the membrane stability of PC/PE-LUV as deduced from the very low leakage of these vesicles (Figure 2C). A similar low release of calcein was also found for vesicles containing PC, PE, and, in addition, cholesterol (Figure 2C). No leakage was observed for liposomes composed of extracted bull sperm lipids in the presence of PDC-109 (Figure 2D).

Next, it was investigated whether an extraction of cholesterol from cholesterol-containing LUV might modulate the impact of PDC-109 on membranes. For that, vesicles were incubated with MCD (10 mM), which extracts cholesterol from membranes (see Experimental Procedures). Upon addition of PDC-109 to these vesicles, leakage was significantly increased to values similar to those found in the absence of cholesterol (Figure 2A, open square). A similar observation was made for PC/SM/cholesterol-LUV (Figure 2B, open square). Using liposomes composed of PC/PE/cholesterol or of lipids extracted from bull spermatozoa, preincubation with MCD resulted only in a slightly increased leakage upon the addition of PDC-109 (Figure 2C,D, open squares). In our measurements, leakage induced by incubation of LUV with MCD alone was typically below 15%.

PDC-109-Induced Permation of Dithionite. To confirm the results on PDC-109-mediated membrane destabilization measured by calcein leakage, we applied another approach, which measures the accessibility of fluorescent lipids in the outer and in the inner membrane leaflet toward dithionite (*51*, *54*). LUVs composed of PC or PC/cholesterol were labeled symmetrically with C6-NBD-PC. Upon addition of sodium dithionite, in the absence of PDC-109, the fluorescence intensity decreased to 45.0 ± 0.8% ($n = 4$) of the initial value due to chemical reduction of the NBD groups localized at the outer leaflet. The amount of fluorescence remaining originates from C6-NBD-PC analogues localized on the inner leaflet of the membrane. When PC-LUV were preincubated with various amounts of PDC-109, a larger decrease of fluorescence intensity was observed after the addition of dithionite; that is, the fraction of accessible analogues increased with higher P/L, indicating a protein-induced permeation of dithionite across the membrane. The extent of dithionite permeation was quantified as described in the Experimental Procedures and is shown in Figure 3. When PC-LUV contained cholesterol (30 mol %), in the presence of PDC-109, no permeation of dithionite was observed up to a P/L of 0.5. Only at the rather high P/L of 1, some increase of permeation was detected, which was significantly lower as compared to PC-LUV, however. Extraction of cholesterol from PC/cholesterol-LUV by incubating vesicles with MCD (10 mM, 10 min) caused an increase of dithionite permeation to a value similar to that measured for PC-LUV (Figure 3, open square, only investigated for P/L = 0.2). We note that for PC/cholesterol-LUV, the fraction of C6-NBD-PC, which was not accessible to dithionite in the absence of PDC-109, was smaller (38.7 ± 2.9%) than for PC-LUV (see above). The data confirm that PDC-109 causes a perturbation of PC membranes, which is reduced in the presence of cholesterol.

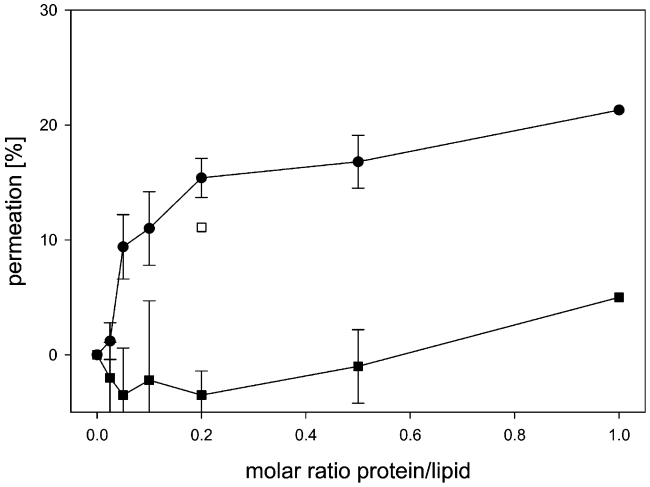

FIGURE 3: PDC-109-induced permation of sodium dithionite to the inner leaflet of LUV. LUVs were symmetrically labeled with C6-NBD-PC, and the dithionite-mediated decrease of fluorescence was measured at a lipid concentration of 25 μM at 20 °C in the presence of various PDC-109 concentrations (see the Experimental Procedures). The percentage of dithionite permeation (see the Experimental Procedures) was determined for PC-LUV (circles) and PC/cholesterol (2:1)-LUV (squares). Additionally, at P/L of 0.2, PC/cholesterol-LUVs were preincubated with 10 mM MCD for 10 min before the addition of PDC-109 (open square). Data represent the means ± SEMs of three independent measurements.

Changes in Intrinsic Fluorescence of PDC-109. Upon interaction with PC-LUV, the intrinsic fluorescence of PDC-109, mainly caused by its five tryptophan residues, increased with an increasing L/P concomitant with a blue shift of the fluorescence maximum (Figure 4). These data, which are in agreement with previous studies (*24*, *26*), indicate a localization of tryptophan residues in a more hydrophobic environment, probably caused by an intercalation of (part of) the protein into the bilayer. When PDC-109 was mixed with liposomes containing PC and cholesterol (30 mol %), we also observed a L/P-dependent increase and blue shift of the fluorescence. However, as compared to PC-LUV, higher lipid (PC + cholesterol) concentrations were necessary to observe similar effects. Whereas for PC/cholesterol-LUV fluorescence intensity and wavelength of fluorescence maximum reached a plateau at L/P = 20, the respective value for PC-LUV was 10.

PDC-109-Induced Hemolysis. To determine the effect of PDC-109 on the integrity of biological membranes, we first used human red blood cells as a model and measured hemolysis of the cells in the presence of the protein. Erythrocytes were incubated at 37 °C with the protein at various P/L ratios, and the amount of hemoglobin leakage was determined after 10 min (Figure 5A). Whereas at low protein concentrations (P/L < 0.2) hemolysis was comparatively low (below 10%), PDC-109 induced considerable leakage of hemoglobin at higher P/L; for example, at P/L = 1, about 50% of the hemoglobin was found in the supernatant. When 46.3 ± 3.1% ($n = 4$) of the erythrocyte cholesterol was extracted by preincubation with 10 mM MCD for 10 min, almost a complete hemolysis was found

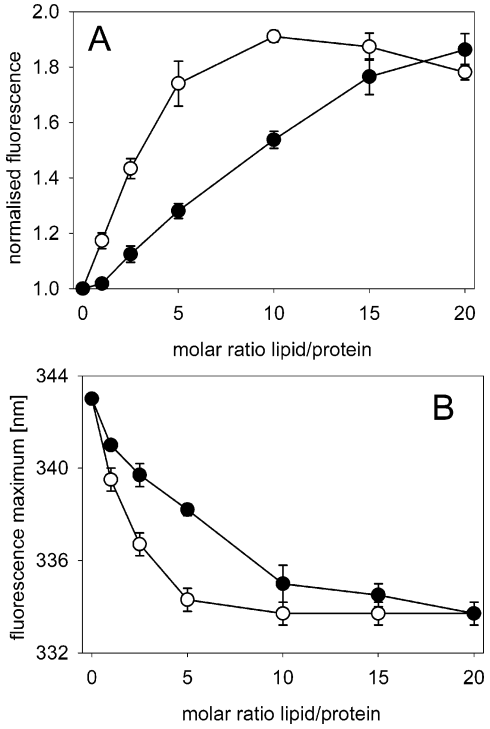

FIGURE 4: Intrinsic fluorescence of PDC-109 in the presence of LUV. PDC-109 (2.5 μM) was incubated with increasing amounts of LUV composed of PC (open circles) or PC containing 30% cholesterol (filled squares), and fluorescence spectra of tryptophan residues were recorded at 37 °C as described in the Experimental Procedures. From fluorescence spectra, the increase in fluorescence intensity (A) and the blue-shift of the fluorescence maximum (B) at different lipid to protein ratios were estimated. In A, fluorescence intensities of PDC-109 in the presence of vesicles were related to the intensity in the absence of vesicles. Data represent the means ± SEMs of three independent measurements.

upon addition of PDC-109 (open square in Figure 5A, only shown for P/L = 0.1). Incubating erythrocytes solely with 10 mM MCD caused a hemolysis of 23.6 ± 4.4% (n = 6). When erythrocytes were incubated with only 3 mM MCD leading to a very weak cholesterol extraction (2.1 ± 0.3%, n = 4), addition of PDC-109 did not cause a significant increase of hemolysis as compared to cells solely incubated with the protein (data not shown).

PDC-109-Induced Perturbation of Sperm Cell Integrity. Second, we tested the influence of PDC-109 and/or MCD on bovine epididymal sperm cells by measuring their PI staining, which is widely used as a parameter for integrity of the plasma membrane. Because of the time-consuming preparation of epididymal sperm cells including several centrifugation steps, a comparatively high amount of these cells was already stained by PI solely incubated in buffer (43.1 ± 5.2%, n = 3). Epididymal sperm cells were incubated with PDC-109 (P/L = 0.1) and/or MCD (5 mM) for 10 min at 38.5 °C, and the proportion of PI stained cells was measured and related to that in the absence of these substances. Incubating sperm cells with PDC-109 did not change the percentage of PI stained cells as compared to control cells (Figure 5B). Similarly, in the presence of MCD alone, the fraction of PI stained cells was not changed (Figure 5B). However, in the presence of both substances, an increase of PI stained cells was observed. Quantification of cholesterol extraction from sperm cells by MCD at these conditions

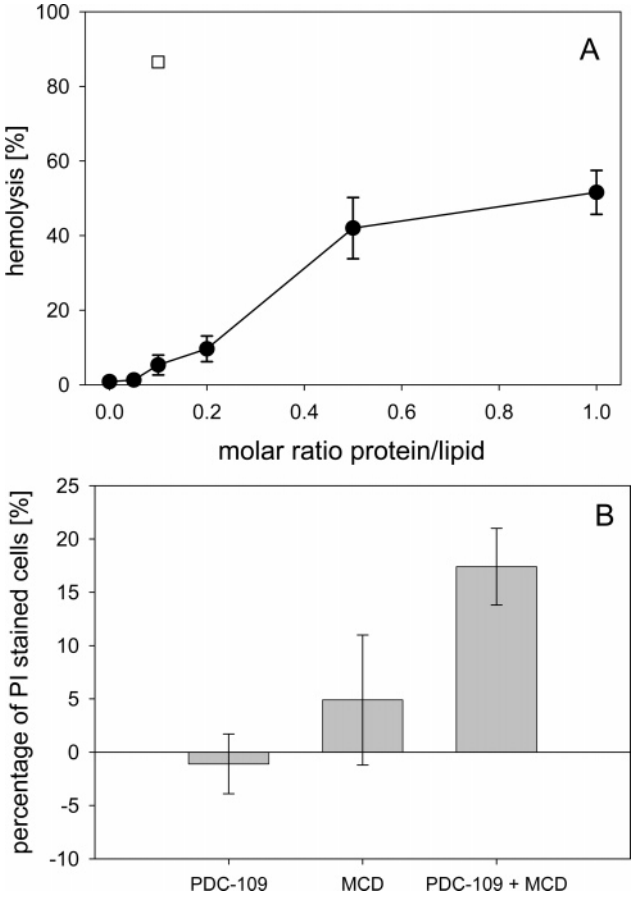

FIGURE 5: PDC-109-induced destabilization of biological membranes. (A) Human erythrocytes with a hematocrit of 10 were incubated with various amounts of PDC-109 for 10 min at 37 °C. Hemolysis was followed by measuring the absorption at 540 nm after pelleting erythrocytes (filled circles) as described in the Experimental Procedures. The open square shows the hemolysis of erythrocytes in the presence of PDC-109 (P/L = 1:10) after preincubation of erythrocytes with 10 mM MCD. Data represent the means ± SEMs of three independent measurements. (B) PI staining of cauda epididymal bull sperm cells after incubation of cells for 10 min at 38.5 °C with PDC-109 (P/L = 1:10), MCD (5 mM), or PDC-109 (P/L = 1:10), and MCD (5 mM) was measured by flow cytometry as described in the Experimental Procedures. The amount of PI stained cells was related to that of control cells, which were solely incubated in buffer. Data represent the means ± SEMs of three independent measurements.

(incubation with 5 mM MCD for 10 min at 38.5 °C) revealed a value of 26.0 ± 7.0% (n = 3).

DISCUSSION

Seminal Fn type II proteins interact with membranes in a complex manner (*18*, *25*, *26*, *28*, *30*−*33*). Previous studies have shown that the bovine protein PDC-109, besides stabilizing membranes (*25*, *28*), is also able to deteriorate the membrane structure (*25*, *26*, *28*). By that, the membrane loses its properties as a barrier allowing, for example, an enhanced permeation of solutes across the bilayer. These data may lead to the conclusion that the interaction of the protein with the plasma membrane could impair bull sperm cells during their genesis. However, those results have been obtained from studies on simple lipid systems (*25*, *26*). The specific lipid composition of the bull sperm plasma membrane may prevent a destabilizing effect of PDC-109. Therefore, in the present manuscript, we compared the

influence of PDC-109 on biological membranes to that on lipid membranes mimicking more closely the lipid composition of the plasma membrane of bull sperm cells.

We found that the lipid composition of membranes influences the degree of perturbation of the bilayer structure by PDC-109. The effective perturbation of membranes containing PC or SM by PDC-109 agrees with the head group specificity of the protein. Previous studies using various approaches have shown that the protein specifically binds to lipids bearing a phosphorylcholine head group, which probably also resemble its binding sites on the bull sperm cell (*23*−*25*). While the protein disturbed membranes solely containing PC and SM, the effect of the protein was considerably diminished in the presence of cholesterol or PE. When membranes containing cholesterol and phospholipids with a phosphorylcholine head group were preincubated with MCD, which is known to extract cholesterol from membranes, PDC-109 disturbed the membrane to a similar extent as found for pure PC vesicles. This underlines that changes of the membrane cholesterol content modulate the impact of PDC-109 (see below). Part of these data are in accordance with other studies, which found that a membrane-destabilizing effect of PDC-109 is reduced in the presence of cholesterol (*25*, *26*). The impact of PDC-109 on lipid membrane structure was characterized by two different approaches, the efflux of calcein from liposomes and the accessibility of fluorescent PC to dithionite, both giving similar results. The larger fluorescence decrease of C6-NBD-PC in the presence of dithionite and PDC-109 (see Figure 3) might be explained by an increase of (i) transbilayer movement of lipids and/or (ii) permeation of dithionite across the membrane, both caused by the protein. We surmise that fluorescence changes are caused by the latter case, since we did not observe an influence of PDC-109 on the transbilayer movement of phospholipids recently (*34*).

The diminished perturbation in PE- and cholesterol-containing membranes can be explained by a decreased binding of PDC-109 to those membranes. It has been shown that the protein has a lower or no affinity to PE and cholesterol, respectively (*23*, *24*, *29*, *55*). In agreement with this, our data employing the intrinsic tryptophan fluorescence upon binding of PDC-109 to LUV indicate a reduced binding of the protein to the membrane in the presence of cholesterol. At a first glance, our data may contradict those of Gasset et al. (*26*). They did not find any effect of cholesterol on binding of PDC-109 to dioleoyl PC vesicles also by measuring the intrinsic tryptophan fluorescence. The discrepancy between Gassets and our results can be explained very likely by the different nature of phospholipids that were used to prepare LUV in both studies, dioleoyl PC containing two unsaturated fatty acids vs PC from egg yolk containing a mixture of saturated and unsaturated fatty acids. Therefore, we measured the impact of cholesterol on intrinsic protein fluorescence using dioleoyl PC-LUVs and confirmed the data of Gassets' study (data not shown). Thus, the fatty acid composition of lipids, which determines the physical properties of the membrane, seems also to influence the impact of PDC-109. Indeed, we have shown that the physical state of lipids is important for the interaction of PDC-109 with membranes, in that the protein more effectively binds to fluid membranes (*28*).

The impact of PDC-109 on biological membranes was investigated on human erythrocytes and on epididymal bull sperm cells. For erythrocytes, the protein caused at low, physiologically relevant protein to lipid ratios (about 1:10) a very low hemolysis. Extraction of cholesterol by MCD before addition of PDC-109 considerably increased protein-mediated membrane disturbation demonstrated by an almost complete hemolysis. Likewise, although PDC-109 binds to epididymal sperm cells (*28*), it did not affect plasma membrane integrity at a protein to lipid ratio of 1:10. When the cholesterol content of sperm cells was reduced by MCD, PDC-109 impaired sperm cell integrity as seen from an increased number of PI-stained cells. These results on biological membranes again underline the role of cholesterol in preventing a destabilization of membranes by PDC-109.

On the other hand, one may argue that other lipids as, for example, PE should diminish the impact of PDC-109 even in the absence of cholesterol. However, it has been shown that PDC-109 interacts with the outer membrane leaflet (*25*, *28*, *34*). Therefore, for understanding the impact of PDC-109 on membrane integrity, the transbilayer distribution of lipids also has to be considered. The outer leaflet of mammalian cells, including sperm cells, mainly contains PC and SM, whereas the aminophospholipids PS and PE are concentrated on the inner monolayer (for a review see, ref *56*). For cholesterol, a more symmetrical distribution between both leaflets has been assumed at least for erythrocytes (*57*). Upon binding to membranes, PDC-109 interacts with the outer, mainly PC/SM/cholesterol-containing leaflet. Because of the presence of cholesterol in this leaflet, membrane integrity is preserved. An extraction of cholesterol would prime the membrane to be more sensitive for an interaction with PDC-109 resulting in a disturbance of membrane structure. On the basis of these results, we surmise that the composition and transbilayer organization of lipids in the plasma membrane of bull sperm cells allow upon ejaculation an effective binding of PDC-109, which, because of the presence of cholesterol, does not cause a perturbation of the membrane integrity.

Our data contribute to the understanding of the role of PDC-109 during sperm cell genesis. The lipid composition of bull sperm cells is modulated during their epididymal transit in that, for example, the cholesterol/phospholipid ratio increases (*58*). Upon ejaculation, PDC-109 effectively binds to the sperm cells without causing a perturbation of their plasma membrane but stabilizing the cells against a premature triggering of capacitation and acrosome reaction (*2*, *26*, *28*). Because (i) the plasma membrane of bull spermatozoa contains besides PC and SM also other phospholipids (PE, phosphatidylserine) and cholesterol and (ii) the transbilayer distribution of these lipids is similar to that in other mammalian cells (*59*), we propose that mainly the presence of cholesterol prevents a PDC-109-induced disturbance upon ejaculation (*26*).

However, locally (at the oviductal binding sites) and temporary (at the time of ovulation) sperm cells have to be destabilized during capacitation to prime them for the acrosome reaction (*60*). To understand the role of PDC-109 for these processes, several effects of the protein on membranes/cells have to be considered as follows: (i) mediation of binding between sperm cells and oviductal epithelium (*61*), (ii) extraction of lipids from membranes

(*31*−*34*), and (iii) destabilization of membranes (*25*, *26*, this study). Recently, it has been shown that PDC-109 promotes the binding of sperm cells to oviductal epithelium forming an oviductal sperm reservoir (*61*). A loss of PDC-109 from sperm cells during capacitation seems to account for a release of sperm from the epithelium. The loss of PDC-109 from sperm cell membrane could be caused by an interaction of the protein with heparin-like glycosaminoglycans (*62*, *63*) or by changes of the sperm membrane composition (*15*), for example, after contact with follicular fluid entering the oviduct with the cumulus mass (*37*, *60*). For the latter point, increased phospholipase A_2 activity, release of membrane cholesterol onto extracellular acceptors (albumin, HDL), and/or release of lipids mediated by PDC-109 itself have to be considered. A modified membrane composition, in particular that of cholesterol, would modulate the interaction of remaining PDC-109 with the sperm membrane, triggering a destabilization of the bilayer structure (*26*). We note that more studies are still required to understand the function(s) of PDC-109 within the sequence of complex processes during capacitation and acrosome reaction. Summarizing, capacitation can be regarded as a controlled destabilization of sperm cells with regard to time and to location (*60*). Seminal Fn type II proteins seem to be a component of this regulatory system.

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