Influence of the Bovine Seminal Plasma Protein PDC-109 on the Physical State of Membranes†

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ABSTRACT: PDC-109 is the main component of bovine seminal plasma and has been suggested to play an important role in the genesis of bovine sperm cells. Here, the effect of binding of PDC-109 to membranes on the structure and physical properties of the lipid phase was investigated. For that, ESR measurements were undertaken on model membranes (lipid vesicles) and on biological membranes (epididymal spermatozoa) by employing various spin-labeled phospholipids. We found that PDC-109 alters the membrane structure of lipid vesicles as well as of bovine epididymal spermatozoa in that the mobility of spin-labeled phospholipids was reduced in the presence of the protein. This immobilizing effect of the protein was not restricted to analogues of phosphatidylcholine but was also detected with spin-labeled phosphatidylethanolamine. However, the extent of immobilization was lower for phosphatidylethanolamine compared with phosphatidylcholine, supporting the lipid headgroup specificity of the protein. Besides phospholipid headgroups, the physical state of membrane lipids is also important for the interaction of PDC-109 with membranes, in that, e.g., the immobilizing effect of the protein on labeled lipids was larger in membranes above the phase transition temperature compared with the effect below this temperature. The results are of relevance for understanding the physiological role of PDC-109 in the genesis of sperm cells.

In the course of their genesis mammalian sperm cells pass different compartments of the male as well as of the female tract. During this passage, distinct steps of sperm genesis, e.g., capacitation and acrosome reaction, take place priming the cell for fertilization. Those steps are modulated or even determined by the extracellular environment of the sperm cell. Upon ejaculation sperm cells come into contact with the seminal plasma which contains a large number of different proteins (*1*). Some of these proteins were shown to have important functions in sperm maturation and fertilization, respectively (*2*−*5*).

Bovine seminal plasma contains four major acidic proteins, designated as BSP-A1, BSP-A2, BSP-A3, and BSP-30K. BSP-A1 and BSP-A2, which have also been termed PDC-109 (*6*) or "major protein" (*7*), have the same polypeptide chain but differ in their glycosylation pattern (*8*, *9*). The first evidence for a physiological role of PDC-109 has been implicated from the work of Manjunath and co-workers (*10*). They show that epididymal spermatozoa which hardly capacitate can be stimulated to undergo this process more rapidly by preincubating the cells with PDC-109. This suggests a regulatory role of the protein during capacitation. Recent research on this protein has been focusing (i) on its structural characterization and (ii) on its physiological impact on sperm cells and, in particular, on fertilization (*11*−*15*). The primary mosaic structure of PDC-109 is characterized by two homologous domains similar to the consensus sequence of the fibronectin type II module (*16*). In aqueous solution PDC-109 organizes into multimeric aggregates depending, e.g., on the ionic strength of the solution (*11*).

To exert a physiological impact, PDC-109 has to interact with the sperm cells. Indeed, Calvete et al. (*8*) estimated that about 9.5 million protein molecules bind to the surface of a bull spermatozoon upon ejaculation. There is strong evidence that the binding of PDC-109 to spermatozoa occurs mainly via phospholipids of the sperm cell plasma membrane, specifically those bearing the phosphorylcholine headgroup (*11*, *15*, *17*, *18*). During capacitation a part of PDC-109 molecules bound to spermatozoa is released from the cells (*8*). This release of PDC-109 seems to mediate an extraction of lipids from the sperm cell membrane since Manjunath and co-workers could show that incubation of epididymal spermatozoa with PDC-109 results in a release of endogenous lipids, mainly phosphatidylcholine (PC)[1] and cholesterol, from the plasma membrane of these cells (*19*). This result supports the physiological role of PDC-109 because a transfer of membrane cholesterol to extracellular cholesterol acceptors (bovine serum albumin, HDL) has been discussed to be one of the crucial steps during capacitation of mammalian sperm cells (*20*−*23*).

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In the present study we have investigated the effect of binding of PDC-109 to membranes on the structure and physical properties of the lipid phase. The study was undertaken on model membranes (large unilamellar vesicles, LUV) and on biological membranes (epididymal spermatozoa). We have employed ESR and various spin-labeled phospholipids (SL-PL) differing in headgroup and fatty acid chain. ESR spectra of SL-PL are sensitive to lipid−protein interactions (*24*, *25*). Moreover, we were interested in whether the physical state of the membrane, e.g., the degree of saturation of fatty acids, modifies protein−membrane interaction. For that we have measured the effect of PDC-109 on SL-PL in membranes of different composition. We found that PDC-109 altered the membrane structure of lipid vesicles as well as of bovine epididymal spermatozoa in that the mobility of spin-labeled phospholipids was decreased. This immobilizing effect of the protein was not restricted to analogues of PC but was also detected with spin-labeled phosphatidylethanolamine (SL-PE). However, the extent of immobilization was lower for SL-PE compared with SL-PC, supporting the lipid headgroup specificity of the protein (*11*, *15*, *17*, *18*). The extent of changes in membrane structure mediated by PDC-109 depended on the physical state of the membrane. The relevance of our results for understanding the role of PDC-109 during capacitation/acrosome reaction is discussed.

MATERIALS AND METHODS

Phospholipids. L-α-Phosphatidylcholine (PC), L-α-phosphatidylethanolamine (PE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and 1-octadecanoyl-2-[(*cis*,*cis*,-*cis*,*cis*,*cis*,*cis*)-4,7,10,13,16,19-docosahexaenoyl]-*sn*-glycero-3-phosphocholine (DSPC) were purchased from Sigma (Deisenhofen, Germany). Short-chain spin-labeled phospholipids (sc-SL-PL) 1-palmitoyl-2-(4-doxylpentanoyl)-*sn*-glycero-3-phosphocholine (sc-SL-PC) and 1-palmitoyl-2-(4-doxylpentanoyl)-*sn*-glycero-3-phosphoethanolamine (sc-SL-PE) were prepared as previously described (*27*). Long-chain spin-labeled phospholipids (lc-SL-PL) 1-palmitoyl-2-stearoyl(5-doxyl)-*sn*-glycero-3-phosphocholine (C5-SL-PC), 1-palmitoyl-2-stearoyl(10-doxyl)-*sn*-glycero-3-phosphocholine (C10-SL-PC), and 1-palmitoyl-2-stearoyl(16-doxyl)-*sn*-glycero-3-phosphocholine (C16-SL-PC) were purchased from Avanti Polar Lipids (Alabaster, AL). Long-chain spin-labeled PE 1-palmitoyl-2-stearoyl(5-doxyl)-*sn*-glycero-3-phosphoethanolamine (C5-SL-PE) was prepared from C5-SL-PC by headgroup exchange (*26*).

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 (*27*). 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. For the ESR measurements (see below) a 2.7 mM stock solution of PDC-109 was prepared in HEPES-buffered saline (HBS, 150 mM NaCl, 5 mM HEPES, pH 7.4).

Preparation and Labeling of Liposomes. Lipids dissolved in chloroform were combined in a glass tube to give the desired composition and concentration. If required, at this stage spin-labeled lipids dissolved in chloroform/methanol (1:1 v/v) were added (2.5 mol % of total lipids), resulting finally in symmetrically labeled liposomes (both leaflets labeled). The mixture was dried under nitrogen. HBS was added to give a final lipid concentration of 8 mM, and the lipids were hydrated by vigorous vortexing. Large unilamellar vesicles (LUV) were prepared using an extruder (Lipex Biomembranes Inc., Vancouver, Canada) with five freeze−thaw cycles and filtration through 0.1 mm pores (10 cycles) at 40 °C (*28*).

For selective labeling of the outer membrane leaflet of LUV (asymmetrically labeled vesicles) with sc-SL-PL, analogues dissolved in chloroform/methanol (1:1 v/v) were transferred to a glass tube, dried under nitrogen, and vortexed with the desired volume of HBS. sc-SL-PL were incorporated into the outer membrane leaflet by mixing the aqueous dispersion of analogues with unlabeled LUV (final analogue concentration 2.5 mol %). We have shown that sc-SL-PL transfer from their aqueous dispersion into membranes within a few seconds (*29*).

Preparation and Labeling of Epididymal Sperm Cells. Bovine testis was purchased from the local slaughterhouse. The epididymis from two animals were dissected according to ref *30*. Spermatozoa from the cauda 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 (800*g*, 10 min), and the pellet was resuspended in 20 volumes of modified Beltsville thawing solution (mBTS) containing 205 mM glucose, 20 mM sodium citrate, 15 mM $NaHCO_3$, 10 mM KCl, 1.1 mM $MgCl_2$, and 1 mM EGTA (pH 7.4). After a second centrifugation, cells were pooled and resuspended in mBTS again to about 1.5×10^9 cells/mL. sc-SL-PC was transferred to a glass tube, dried under nitrogen, and vortexed with the desired volume of mBTS. Sperm cells were labeled with sc-SL-PC for 5 min at 25 °C (final label concentration was about 2% of endogenous membrane phospholipids) and, subsequently, washed once with mBTS to discard non-incorporated analogue. Labeled sperm cells were mixed with mBTS or PDC-109 for 5 min at 25 °C. The final molar ratio of protein to membrane lipids was about 1:20 assuming a lipid content of 1.7 μmol for the plasma membrane of 10^9 sperm cells (*31*). The P/L ratio was chosen in order to meet the physiological conditions with regard to the concentration of PDC-109 in seminal plasma as well as to the amount of

[1] Abbreviations: C5-SL-PC, 1-palmitoyl-2-stearoyl(5-doxyl)-*sn*-glycero-3-phosphocholine; C5-SL-PE, 1-palmitoyl-2-stearoyl(5-doxyl)-*sn*-glycero-3-phosphoethanolamine; C10-SL-PC, 1-palmitoyl-2-stearoyl-(10-doxyl)-*sn*-glycero-3-phosphocholine; C16-SL-PC, 1-palmitoyl-2-stearoyl(16-doxyl)-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSPC, 1-octadecanoyl-2-[(*cis*,*cis*,*cis*,*cis*,-*cis*,*cis*)-4,7,10,13,16,19-docosahexaenoyl]-*sn*-glycero-3-phosphocholine; egg PC, egg lecithin; ESR, electron spin resonance spectroscopy; HBS, HEPES-buffered saline; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*-2-ethanesulfonic acid; lc-SL-PL, long-chain spin-labeled phospholipid(s); LUV, large unilamellar vesicle(s); mBTS, modified Beltsville thawing solution; PC, L-α-phosphatidylcholine; PE, L-α-phosphatidylethanolamine; PL, phospholipid(s); P/L, molar ratio of protein to lipid; sc-SL-PC, short-chain C5-spin-labeled phosphatidylcholine [1-palmitoyl-2-(4-doxylpentanoyl)-PC]; sc-SL-PE, short-chain C5-spin-labeled phosphatidylethanolamine [1-palmitoyl-2-(4-doxylpentanoyl)-PE]; sc-SL-PL, short-chain spin-labeled phospholipid(s); SL-PL, spin-labeled phospholipid(s); SUV, small unilamellar vesicle(s); T_C, phase transition temperature.

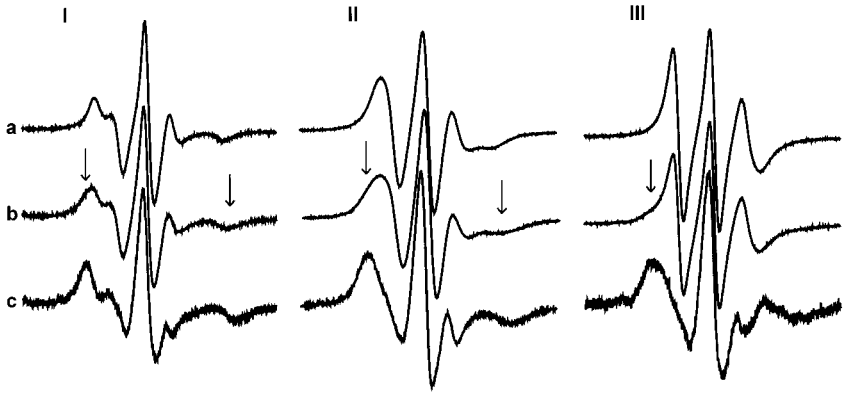

FIGURE 1: Influence of PDC-109 on the ESR spectra of different long-chain spin-labeled analogues of phosphatidylcholine incorporated into egg PC LUV membranes. Egg PC LUV (2 mM) were labeled symmetrically with 0.05 mM C5-SL-PC (I), C10-SL-PC (II), and C16-SL-PC (III). The ESR spectra were recorded at 25 °C (I, II) or at 4 °C (III) in the absence (a) and in the presence (b) of 0.2 mM PDC-109. In the presence of the protein an immobilized component was observed (see arrows), which was extracted by spectra subtraction [(b) − (a)] yielding spectra shown in (c).

protein bound to sperm cells (*8*). ESR spectra of cells were recorded as described below in the presence of 10 mM sodium hexacyanoferrat in order to keep the analogue oxidized. Subsequently, cells were withdrawn from the capillaries and after washing spermatozoa once in mBTS spectra were recorded again.

ESR Measurements. Labeled LUV and sperm cells, respectively, were mixed with PDC-109 and incubated for 5 min on ice (LUV) or at 25 °C (sperm cells). The final lipid concentration of LUV was 2 mM. ESR spectra were recorded at 25 °C for long-chain analogues (if not stated otherwise) or at 4 °C for short-chain analogues in liposomes. These temperatures were chosen in order to allow deconvolution of immobile spectral components due to protein−lipid interaction. When the spectra were recorded at the physiological temperature of 39 °C, this convolution was hampered owing very likely to the rapid exchange of labeled lipids between the bulk lipid phase and the protein neighborhood. ESR spectra of sc-SL-PC in epididymal sperm cells were recorded at 25 °C in order to prevent low-temperature induced deterioration of cell integrity. ESR spectra were run at a Bruker ECS 106 spectrometer (Bruker, Karlsruhe, Germany) with the following parameters: modulation amplitude 2.5 G, power 20 mW, scan width 100 G, and accumulation 16 times (for LUV) and 4 times (for sperm cells).

To obtain the spectra of the immobilized component in the presence of PDC-109, we have subtracted the ESR spectra in the absence of protein from those in the presence of protein using standard ESR software (Bruker, Karlsruhe, Germany). The resulting spectra represent those of the immobilized analogue molecules. For a quantitative comparison of the ESR spectra two parameters were used. First, the degree of immobilization, i.e., the amount of spin-labeled molecules affected by the protein, was estimated from spectra subtraction (see above). We are aware that this quantification might be a simplification of the PDC-109−membrane interaction since it assumed that only two states of lipids exist in the presence of the protein and that the exchange rate between these states is negligible at the time scale of ESR. Second, as a measure of the motional restriction of spin-labeled analogues the outer hyperfine splitting ($2A_{max}$) was estimated from the ESR spectra.

All spectra shown were normalized to the same double integral.

Ascorbat Assay. LUV (2 mM) symmetrically labeled with 0.05 mM C5-SL-PC (see above) were incubated for 5 min with (P/L = 1:10) and without PDC-109. Subsequently, ascorbic acid in HBS was added (final concentration 20 mM) from a stock solution adjusted to pH 7.4. ESR spectra were recorded at 25 °C (egg PC and DSPC LUV) or at 45 °C (DPPC LUV) at different times using a Bruker ECS 106 spectrometer (see above, same parameters but without accumulation). After 20 min the samples were withdrawn from the ESR capillaries, Triton X-100 was added (final concentration 1%), and again ESR spectra were recorded. The decrease of ESR signal intensity was estimated by relating the double integrals of ESR spectra to those in the absence of ascorbic acid. From all spectra the double integral of the baseline (spectrum of HBS) was subtracted.

RESULTS

Interaction of PDC-109 with Egg PC LUV. (A) Long-Chain Labeled PC (lc-SL-PC). To exclude any effect of surface curvature on the protein−membrane interaction, we have investigated the interaction of PDC-109 with large unilamellar vesicles (LUV). To measure the mobility of lipids, we employed long-chain spin-labeled phospholipids (lc-SL-PL) having a long-chain fatty acid at both the *sn*-1 and *sn*-2 positions (the latter one bearing the doxyl moiety), which are suitable analogues for endogenous phospholipids. lc-SL-PL were added before preparation of LUV (see Materials and Methods), resulting in symmetrically labeled membranes; i.e., analogues are localized on both (inner and outer) membrane leaflets. By using PC analogues bearing the label moiety at different positions of the fatty acid chain (C5-SL-PC, C10-SL-PC, C16-SL-PC), various depths of the hydrophobic membrane core can be sensed. The spectra of these analogues reflect a gradient of motional freedom along the fatty acid residues in membranes, with the region near the headgroups being more immobilized and the flexibility increasing toward the terminal methyl groups. In agreement with this, the spectra of C5-SL-PC, C10-SL-PC, and C16-SL-PC in egg PC LUV at 25 °C indicated an increasing motional freedom of the respective analogue (Figure 1a, only shown for C5-SL-PC and C10-SL-PC).

Table 1: PDC-109-Mediated Immobilization of Spin-Labeled Lipids in LUV and Epididymal Sperm Membranes and the Respective Outer Hyperfine Splitting Values[a]

membrane[b]	analogue[c]	extent of immobilization (%)	$2A_{max}$ (G)	
			−PDC	+PDC
egg PC, 25 °C	C5-SL-PC, sym	48	51.1	60.2
	C10-SL-PC, sym	48	42.4	56.7
	C5-SL-PE, sym	29	52.1	59.6
egg PC, 4 °C	C16-SL-PC, sym	21	34.1	57.7
	sc-SL-PC, sym	41	34.9	62.5
	sc-SL-PC, asym	86	34.8	61.4
	sc-SL-PE, asym	77	35.6	61.6
DPPC, 45 °C	C5-SL-PC, sym	77	47.2	57.8
DSPC, 25 °C	C5-SL-PC, sym	49	53.7	61.3
DSPC, 25 °C (*)		65	53.4	60.7
cauda, 25 °C (**)	sc-SL-PC, asym	52	35.6	58.5

[a] The extent of lipids immobilized was estimated by subtraction of the ESR spectra in the absence from those in the presence of the protein (P/L = 1:10, except (*) and (**) where P/L = 1:5 and 1:20, respectively; see Materials and Methods). The outer hyperfine splitting $2A_{max}$ was estimated from the spectra in the absence (−PDC) and in the presence of PDC-109 (+PDC). The data represent the means of at least two separate experiments. [b] ESR spectra were recorded from membranes of large unilamellar vesicles of the given lipid composition or of epididymal sperm cells from the cauda at the temperature indicated. [c] Membranes were labeled with the respective analogue either symmetrically (sym, i.e., both leaflets labeled) or asymmetrically (asym, i.e., only the outer leaflet labeled).

In the presence of PDC-109 (molar ratio of protein to lipid, P/L = 1:10) ESR spectra of lc-SL-PC incorporated into egg PC LUV revealed the occurrence of an additional immobilized component which was especially obvious in the region of the low-field peak and in the localization of the high-field peak (Figure 1, only shown for C5-SL-PC and C10-SL-PC; see arrows in spectra b). An immobilized component was observed for all three analogues used (not shown for C16-SL-PC). However, the degree of immobilization in the presence of PDC-109 was dependent on the localization of the label moiety at the fatty acid chain. The broadening of the low-field peak was much more expressed for C5-SL-PC and C10-SL-PC compared with C16-SL-PC. To extract and characterize the immobilized component also on a quantitative level, we subtracted the spectra in the absence from those in the presence of the protein (see Materials and Methods). The respective spectra of the immobilized component of C5-SL-PC and C10-SL-PC are shown in Figure 1c. Since for C16-SL-PC the degree of immobilization in the presence of PDC-109 was too small, we could not perform spectra subtraction for this analogue at 25 °C. However, recording ESR spectra at 4 °C, we could find a substantial immobilized component for C16-SL-PC in the presence of PDC-109 (P/L = 1:10) (Figure 1, IIIc). From spectra subtraction the amount of analogue molecules immobilized in the presence of the protein was estimated and is given in Table 1. For C5-SL-PC and C10-SL-PC about half of the amount of analogue molecules were immobilized in the presence of PDC-109 at 25 °C, suggesting that solely the lipids in the outer membrane leaflet were affected. For C16-SL-PC about 21% of analogue molecules were immobilized at 4 °C.

To estimate the mobility of the analogue molecules in the presence of PDC-109, we measured the outer hyperfine splitting ($2A_{max}$) of the immobilized component, which was about 60.2 and 56.7 G for C5-SL-PC and C10-SL-PC, respectively, at 25 °C. The value of $2A_{max}$ in the absence of protein was about 51.1 and 42.4 G for C5-SL-PC and C10-SL-PC, respectively. For C16-SL-PC at 4 °C the value of $2A_{max}$ was 34.1 G (without protein) and 57.7 G (with protein). From these data, we conclude that PDC-109 causes an effective restriction of the mobility of lipids in the bilayer.

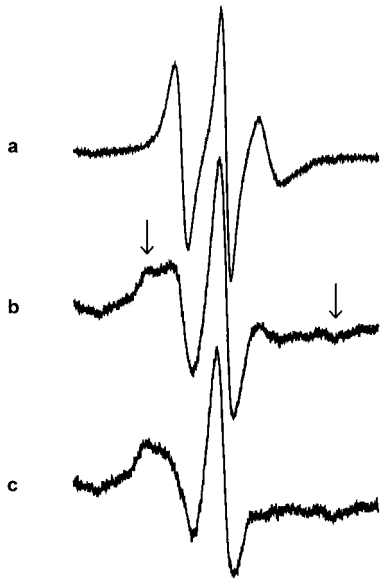

FIGURE 2: Influence of PDC-109 on the ESR spectra of short-chain spin-labeled PC incorporated into the outer leaflet of egg PC LUV membranes. The outer leaflet of 2 mM egg PC LUV was labeled with 0.05 mM sc-SL-PC, and ESR spectra were recorded at 4 °C in the absence (a) and in the presence (b) of 0.2 mM PDC-109. The immobilized component in the presence of the protein (see arrows) was extracted by spectra subtraction yielding spectrum c.

(B) Short-Chain Labeled PC (sc-SL-PC). For investigating the influence of PDC-109 on plasma membranes of epididymal sperm cells, sc-SL-PC, having a short fatty acid with the doxyl group at the *sn*-2 position, was employed (see below). To exclude that the length of the fatty acid chain at the *sn*-2 position of analogues determines the interaction with PDC-109, the effect of the protein on membrane structure detected by sc-SL-PC was first studied on model membranes and compared with that of the respective long-chain analogue (C5-SL-PC).

Egg PC LUV were labeled symmetrically with sc-SL-PC, and ESR spectra were recorded in the absence and in the presence of PDC-109. As observed for lc-SL-PC, upon addition of PDC-109 to labeled LUV (P/L = 1:10), a second, strongly immobilized component in the ESR spectrum was observed (spectra not shown). From spectra subtraction the amount of analogue molecules immobilized was calculated to be about 41%, suggesting that essentially the analogue molecules in the outer membrane leaflet were affected. The outer hyperfine splitting $2A_{max}$ of the immobilized component was about 34.9 G in the absence and 62.5 G in the presence of PDC-109. From these results it can be concluded that sc-SL-PC detects the impact of PDC-109 on lipid membrane similar to C5-SL-PC.

In another set of experiments, sc-SL-PC was incorporated solely into the outer membrane leaflet of egg PC LUV, and ESR spectra were recorded (Figure 2). Again, an immobilized component in the ESR spectrum occurred in the presence of PDC-109 (P/L = 1:10) with about 86% of analogue

molecules immobilized (see Table 1). The outer hyperfine splitting $2A_{max}$ of the immobilized component (Figure 2c) was 34.8 G (without protein) and 61.4 G (with protein), respectively. These data underline the suggestion that at this protein concentration most of the phospholipids in the outer leaflet are affected by PDC-109.

(C) Spin-Labeled PE (C5-SL-PE, sc-SL-PE). Several lines of evidence indicate that the interaction of PDC-109 with membranes is realized via the binding of the protein to lipids containing a phosphorylcholine headgroup (*11*, *15*, *17*). With regard to the effect of the protein on spin-labeled PC analogues, we were interested in whether PDC-109 also might affect the mobility of other lipid species. For that we employed spin-labeled short-chain as well as long-chain analogues of PE, sc-SL-PE and lc-SL-PE, respectively. Recently, we have shown that PDC-109 does directly interact with sc-SL-PC but not with sc-SL-PE in aqueous buffer as a model system (*15*).

Egg PC LUV were labeled with C5-SL-PE (symmetrically labeled) or with sc-SL-PE (asymmetrically labeled), and ESR spectra were recorded in the absence and presence of PDC-109 (P/L = 1:10) similar to the procedure with PC analogues (see above). Remarkably, PDC-109 also caused an immobilization of C5-SL-PE (spectra not shown). The shape of the spectrum of the immobilized component obtained after spectra subtraction was similar to that of the respective PC analogue C5-SL-PC. This was underlined by similar values for the outer hyperfine splitting $2A_{max}$ (C5-SL-PE in the absence and in the presence of protein, 52.1 and 59.6 G, respectively; C5-SL-PC: see Table 1). The amount of C5-SL-PE molecules immobilized (29%) was lower than that of C5-SL-PC—hardly detectable and at the limit of resolution (Table 1). Likewise, PDC-109 mediated an immobilization of sc-SL-PE incorporated into the outer leaflet of egg PC LUV (spectra not shown). Again, the shape of the spectrum of the immobilized component was similar to that observed for sc-SL-PC with a comparable $2A_{max}$ (61.6 G; see Table 1). However, the degree of immobilization of sc-SL-PE in the presence of PDC-109 was lower than that of sc-SL-PC (Table 1). These results support the view that the protein shows a more effective interaction with PC than with PE (*15*, *17*).

Role of the Physical State of Lipids for the Interaction of PDC-109 with Membranes. Compared with other plasma membranes, sperm cell membranes are characterized by an unusual high proportion of phospholipids bearing highly unsaturated fatty acids; e.g., in the ram, bull, and boar about 60% of phospholipid-bound fatty acids are 20:4, 22:5, and 22:6, respectively (*31*−*33*). This unique membrane composition of sperm cells, resulting in a high mobility of membrane constituents, seems to have an important physiologically relevance (*34*). Therefore, we studied whether the physical state of membrane lipids might modulate the impact of PDC-109 on membranes.

LUV composed of fully saturated DPPC were labeled symmetrically with C5-SL-PC, and membrane spectra were recorded below (25 °C) and above (45 °C) the phase transition temperature (T_C) of DPPC membranes. The mobility of lipids is low below T_C and increases considerably at the temperature. In line with this, the hyperfine splitting value $2A_{max}$ of C5-SL-PC is lower at 45 °C (47.2 G) compared with that at 25 °C (60.7 G), respectively. Upon addition of

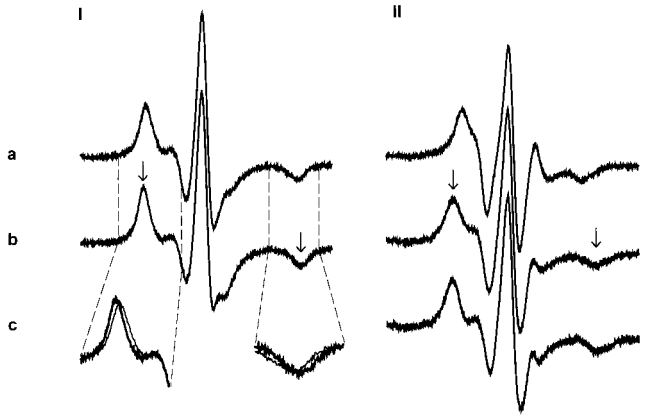

FIGURE 3: Influence of the temperature on the interaction of PDC-109 with DPPC membranes. DPPC LUV (2 mM) were labeled symmetrically with 0.05 mM C5-SL-PC. The ESR spectra were recorded at 25 °C (I) or at 45 °C (II) in the absence (a) and in the presence (b) of 0.02 mM PDC-109. The immobilized component in the presence of the protein (see arrows) at 45 °C was extracted by spectra subtraction yielding spectrum shown in (IIc). Since the degree of immobilization at 25 °C was too small, spectra subtraction could not be performed. In (Ic) the low-field and the high-field peaks of the spectra at 25 °C without (thin solid line) and with protein (bold solid line) were superimposed.

PDC-109 (P/L=1:10), the ESR spectra at both temperatures revealed an immobilization of analogue molecules (Figure 3b).

However, below the T_C binding of PDC-109 to DPPC membranes does not strongly affect the motility of the already immobilized analogue (see the enlarged low- and high-field peaks in Figure 3, Ic). Since the degree of immobilization at that temperature was too small, we could not perform spectra subtraction. Above the T_C PDC-109 mediated an effective immobilization of C5-SL-PC. The spectrum of the immobilized analogue obtained after spectra subtraction is shown in Figure 3, IIc. PDC-109 caused a decrease of the mobility of part of C5-SL-PC at 45 °C as seen from the value of $2A_{max}$, 57.8 G (Table 1). The amount of immobilized molecules above the T_C—which was about 77%—indicates that at this condition also part of the analogues in the inner membrane leaflet are affected by the protein.

In a second approach the influence of PDC-109 on a highly unsaturated lipid was investigated. For that, LUV composed of DSPC (six double bonds) were symmetrically labeled with C5-SL-PC, and ESR spectra were recorded at 25 °C in the absence and in the presence of the protein. PDC-109 (P/L = 1:10) also significantly affected the motility of C5-SL-PC in DSPC LUV in that about 49% of the analogue was immobilized by the protein (Table 1, spectra not shown). At higher protein concentrations the extent of immobilization of C5-SL-PC was more than 50%, similar to what we have found with DPPC membranes above the T_C; e.g., at a P/L of 1:5 about 65% of C5-SL-PC was immobilized in DSPC vesicles. This indicates an influence of the protein also on lipids in the inner membrane leaflet.

One may argue that at those conditions where more than 50% of the analogue is affected, PDC-109 disturbs the lipid bilayer structure leading also to an immobilization of lipids (originally) on the inner leaflet. Additional experimental support for PDC-109-mediated bilayer perturbation, in particular for lipids on the inner leaflet, was obtained from

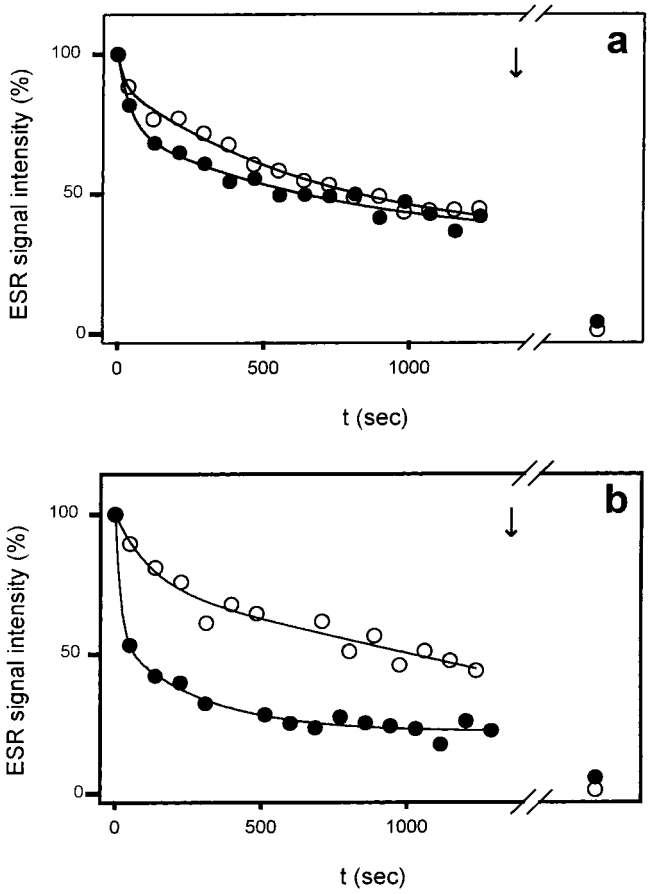

FIGURE 4: Kinetics of ascorbate reduction of C5-SL-PC in LUV membranes. LUV (2 mM) consisting of egg PC (A) or DSPC (B) were symmetrically labeled with 0.05 mM C5-SL-PC. Labeled LUV were mixed with ascorbic acid to give a final concentration of 20 mM at time zero and transferred to an ESR capillary. ESR spectra were recorded at the given time points at 25 °C in the absence (open circles) and in the presence (filled circles) of PDC-109 (P/L = 1:10). ESR signal intensities were estimated by double integration of spectra and were normalized to those in the absence of ascorbate. Spectra were corrected for baseline, i.e., spectrum of HBS. Data were fitted to a biexponential equation (see lines). After 20 min the samples were withdrawn from the ESR capillaries, Triton X-100 was added to a final concentration of 1% (see arrows), and again ESR spectra were recorded.

studying the accessibility of ascorbic acid to C5-SL-PC in symmetrically labeled LUV. Ascorbic acid reduces the NO group of the label moiety, resulting in a loss of the ESR signal. In the absence of PDC-109, upon addition of ascorbic acid (final concentration 20 mM) to egg PC LUV at 25 °C the ESR signal intensity decreased within 10 min to about 50% of the initial intensity (Figure 4a). This decay is related to the reduction of analogues in the outer membrane leaflet. Subsequently, ESR signals decreased more slowly due to a slow permeation of ascorbic acid and/or a slow movement of C5-SL-PC from the inner to the outer leaflet. In the presence of PDC-109 (P/L = 1:10) the reduction of C5-SL-PC in egg PC LUV was only slightly enhanced (Figure 4a), suggesting that the protein does not perturbate the lipid bilayer structure at these conditions.

The kinetics of reduction of C5-SL-PC in DSPC LUV at 25 °C without PDC-109 was similar to that in egg PC LUV (Figure 4). However, in the presence of the protein (P/L = 1:10) the reduction of the analogue in DSPC-LUV was different. Upon addition of ascorbic acid the ESR signal intensity of C5-SL-PC rapidly decreased to values below 50% (Figure 4b). Likewise, we observed a similar rapid reduction of C5-SL-PC in DPPC-LUV at 45 °C in the presence of PDC-109 (P/L = 1:10) (not shown). From these results we surmise that PDC-109 causes a destabilization of the bilayer structure in highly fluid membranes, i.e., DPPC membranes above the T_C or DSPC membranes. However, the bilayer structure was partly preserved since not all analogues were rapidly reduced in the presence of the protein (Figure 4b, only shown for DSPC-LUV). We observed a complete and rapid reduction of C5-SL-PC by ascorbic acid (i) after addition of Triton X-100 (final concentration 1%) to labeled LUV (see Figure 4) or (ii) upon mixing labeled egg PC LUV with mellitin (P/L = 1:20), which has been shown to destabilize membrane structure (*35*).

Interaction of PDC-109 with Plasma Membranes of Bovine Epididymal Sperm Cells. The data obtained above refer to PDC-109-mediated changes in the structure of pure lipid membranes. Most importantly, it is necessary to investigate whether PDC-109 has similar effect(s) on the biological relevant membrane, i.e., on plasma membranes of bovine epididymal sperm cells. For those measurements long-chain labeled analogues are inappropriate since they incorporate very slowly into membranes. This problem can be circumvented by using short-chain spin-labeled phospholipids (sc-SL-PL) because these analogues incorporate into membranes within seconds (*29*). As shown above, short-chain analogues detected PDC-109-mediated membrane changes similar to long-chain analogues. Therefore, sc-SL-PC was used to investigate the impact of PDC-109 on sperm cell membranes.

Membrane spectra of sc-SL-PC incorporated into the outer leaflet of bovine epididymal spermatozoa from the cauda in the absence and in the presence of PDC-109 are shown in Figure 5. The occurrence of the additional component in the ESR spectrum reveals that binding of PDC-109 to the cells caused an immobilization of the analogue also in these membranes (see Figure 5b). From spectra subtraction (Figure 5c) we estimated about 52% of sc-SL-PC molecules to be affected by the protein (Table 1). To investigate whether these PDC-109-mediated changes in the ESR spectra reflect (i) alterations of the mobility of analogues in the membrane or (ii) lipids extracted from membranes and bound to PDC-109 (*13*, *19*), cauda sperm cells labeled with sc-SL-PC were incubated with PDC-109 and, subsequently, washed with PBS to discard protein not bound to the sperm cell membrane (see Materials and Methods). Membrane spectra of those washed cells still contain the immobile component shown in Figure 5c, suggesting that the immobilizing effect of PDC-109 on lipids reflects a mechanism at/in the membrane.

DISCUSSION

The seminal plasma protein PDC-109 has been suggested to play an important role in the genesis of bovine sperm cells (*10*). To understand the physiological effect(s) of this protein, its impact on membranes has to be characterized. It is generally accepted that PDC-109 mainly interacts with phospholipids bearing a phosphorylcholine headgroup (*11*, *15*, *17*, *18*). The aim of the present study was to investigate the effect of PDC-109 on membrane structure, i.e., on the mobility of phospholipids by employing spin-labeled ana-

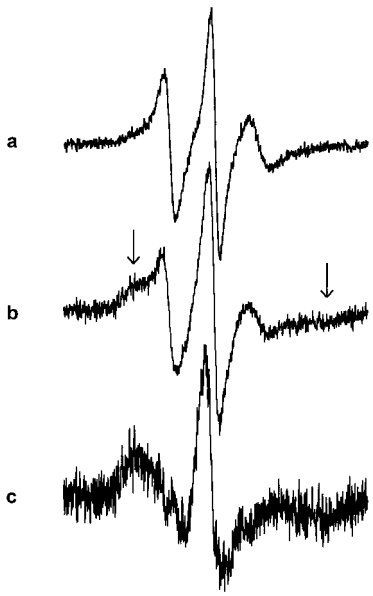

FIGURE 5: Influence of PDC-109 on the ESR spectra of short-chain spin-labeled PC incorporated into bovine epididymal sperm cells from the cauda. Sperm cells were isolated and labeled as described in Materials and Methods. ESR spectra were recorded at 25 °C in the absence (a) and in the presence (b) of PDC-109. The molar ratio of protein to membrane lipids was 1:20. The immobilized component in the presence of PDC-109 (see arrows) was extracted by spectra subtraction yielding spectrum c.

logues of lipids. We were also interested in (i) whether the physical state of the membrane modulates the impact of PDC-109, (ii) whether also lipids other than PC might be affected by the protein, and (iii) whether an effect of PDC-109 on membrane structure of bovine epididymal sperm cells can be detected.

Our study shows that PDC-109 affects the structure of lipid and biological membranes in that the mobility of spin-labeled analogues of PC is decreased in membranes of LUV as well as in the plasma membrane of epididymal spermatozoa. A preferential impact of the protein on the outer membrane leaflet is supported by the results on egg PC LUV, which were symmetrically labeled with C5-SL-PC or sc-SL-PC; PDC-109 usually induced an immobilization of about 50% of analogue molecules, indicating an influence on mainly those molecules incorporated into the outer membrane leaflet. We also detected an immobilization of spin-labeled analogues of PE in the presence of PDC-109. This shows that in a membrane also other phospholipids than PC are affected by the protein. However, the extent of immobilization of PE analogues was significantly lower compared to that obtained with PC. Apart from phospholipid headgroup, the physical state of membrane lipids is also important for the interaction of PDC-109 with membranes, in that the effect of the protein on labeled lipids was larger in membranes above the phase transition temperature or in a highly unsaturated membrane.

The targets of PDC-109 are mainly phospholipids exposed to the outer leaflet. Comparing the effect of PDC-109 on the different long-chain analogues of PC, we found a decrease in the extent of immobilization in the range C5-SL-PC $\sim$ C10-SL-PC $>$ C16-SL-PC. These differences argue for a stronger interaction of PDC-109 with the region near the headgroups of the membrane, which is in line with recent experiments (*15*). However, we do not know whether the lower degree of immobilized analogues of C16-SL-PC indeed reflects a lesser influence of PDC-109 on deeper hydrophobic parts of the lipid bilayer or is a consequence of the lower temperature. However, it shows unambiguously that PDC-109 affects also this part of the bilayer.

Remarkably, binding of PDC-109 to LUV does not only influence spin-labeled PC but also analogues of PE detect an immobilization. So far, no specific interaction between PDC-109 and PE has been demonstrated (*15*, *17*). Here we detected that sc-SL-PE incorporated into a PC-containing membrane is partially immobilized in the presence of PDC-109. Likewise, a long-chain analogue of PE (C5-SL-PE) detects a protein-mediated restriction in its mobility, too. However, the extents of immobilization were less expressed compared with the respective PC analogues (see Table 1). First, these results underline the specificity of PDC-109 for PC lipids (*11*, *15*, *17*, *18*). Second, the data indicate that PDC-109 causes an efficient immobilization of the whole PC-containing membrane which is also detected by PE. Recently, we could show that PDC-109 also mediates an immobilization of spin-labeled analogues of cholesterol dependent on the presence of PC (Müller et al., unpublished experiments). We surmise that the lower fraction of immobilized SL-PE supports the view that PE is not directly affected by PDC-109 but that the immobilization of PC by the protein affects the mobility of neighboring PE molecules.

The influence of PDC-109 on membranes depends on the physical state of the lipid bilayer. The impact of the protein was different on DPPC membranes below and above the phase transition temperature. While at 25 °C only a low amount of C5-SL-PC was immobilized, at 45 °C about 77% of the analogues were motionally restricted. Moreover, in membranes of DSPC vesicles PDC-109 caused an immobilization of $\geq$50% of analogue molecules depending on the protein concentration. From these data it can be concluded that in a highly fluid membrane also parts of lipids of the inner membrane leaflet were affected in the presence of the protein. Several reasons may account for this observation. The lower packing density of lipids at these conditions supports a more effective binding of the protein at the membrane, thereby influencing also the inner leaflet. The ascorbate-mediated reduction kinetics of C5-SL-PC in DPPC LUV (above the T_C) or in DSPC LUV may indicate the formation of porelike structures of PDC-109 in those membranes. This would cause (i) a permeation of ascorbic acid and/or (ii) an increase of the transverse movement of lipids which could explain the enhanced reduction of the analogue in the presence of PDC-109. It has been shown that membrane active peptides such as mellitin and magainin 2 perturbate bilayer structure by forming porelike aggregates within the membrane, e.g., causing a scrambling of lipids (*35*−*37*). Further studies should clarify whether PDC-109 may intercalate into the hydrophobic part of the membrane (*15*). Second, to match the higher packing density and, thus, the reduced surface area of the outer leaflet upon binding of PDC-109, the packing density of the inner leaflet becomes also enhanced. Whatever the reason, the unique lipid composition of mammalian sperm cells, in this case from bovine origin, with a high amount of unsaturated fatty acids (*31*), should ensure an effective interaction of PDC-109 with these plasma membranes.

Indeed, we could show here that PDC-109 causes an immobilization of lipids not only in pure lipid membranes

but also in the relevant biological membrane, i.e., of epididymal sperm cells. For those investigations long-chain analogues are inappropriate because of their slow incorporation into membranes. To characterize the interaction of PDC-109 with the membranes of epididymal spermatozoa, we have employed a short-chain labeled analogue of PC (sc-SL-PC) because of several reasons. First, short-chain labeled (sc-SL-PC) and long-chain labeled analogues (C5-SL-PC) detected the influence of PDC-109 on lipid membranes in a comparable manner. Second, sc-SL-PC incorporates rapidly (within seconds) into membranes, thereby selectively labeling the outer leaflet (*29*). Third, within the time course of our experiments sc-SL-PC resides mainly in the outer leaflet since the analogue traverses plasma membranes slowly by passive diffusion (see ref *38*). In agreement with this we estimated that less than 10% of sc-SL-PC had moved to the inner membrane leaflet of epididymal spermatozoa from the cauda after 5 min at 25 °C as measured by the back-exchange assay (*39*). Additionally, to ensure the orientation of sc-SL-PC mainly in the outer membrane leaflet during our experiments, the number of accumulated spectra was kept low (see Materials and Methods). We found that about 50% of sc-SL-PC molecules were immobilized in cells from the cauda in the presence of PDC-109. In contrast to the PC analogue, spin-labeled aminophospholipids, such as sc-SL-PE, are rapidly transported from the outer to the inner membrane leaflet in various mammalian plasma membranes, including sperm cells, due to the action of the aminophospholipid translocase (*39*−*41*; see ref *38*). In line with this we found that about 70% of sc-SL-PE had moved to the inner membrane leaflet of epididymal spermatozoa from the cauda after 5 min at 25 °C (*39*). Because of this, we did not investigate the influence of PDC-109 on sc-SL-PE in epididymal sperm cells.

Recently, Manjunath and co-workers (*13*, *19*) found that, upon incubation of PDC-109 with epididymal sperm cells or fibroblasts (as model system), the protein extracts lipids, mainly PC and cholesterol, from the plasma membrane, resulting in the formation of HDL-like lipid−protein aggregates. It might be surmised that our results reflect a PDC-mediated extraction at least of the analogue molecules from the membranes. We put special emphasis to this possible explanation of our data. In the experiments using lipid membranes we labeled the LUV with long-chain lipid analogues. These analogues are tighter anchored in the membrane and are hardly extractable, e.g., onto bovine serum albumin. Moreover, the half-time of lipid extraction in the experiments of Manjunath and co-workers was about 10 min at 37 °C (*13*). In our studies LUV labeled with lc-SL-PC were incubated with PDC-109 at 25 °C only for a few minutes. Therefore, extraction of analogues onto PDC-109 should not interfere with our results. Short-chain labeled lipid analogue can be more easily extracted from membranes, e.g., onto bovine serum albumin (*42*). However, ESR spectra of an aqueous solution of sc-SL-PC and PDC-109 (*15*) are quite different from those of sc-SL-PC labeled LUV in the presence of PDC-109 (see above). Therefore, also for LUV labeled with sc-SL-PC the protein-mediated immobilization should not reflect an extraction of analogues. In the experiments on epididymal sperm cells, ESR spectra were additionally recorded after washing cells which were labeled with sc-SL-PC and incubated with PDC-109. Thereby, the protein-mediated changes, observed on lipid and plasma membrane immediately after addition of PDC-109 should reflect alterations of the membrane lipid phase caused by the protein. Our conclusion that the PDC-109-mediated influence on spin-labeled analogues is associated with the membrane lipid phase is not in contrast to the ability of the protein to extract lipids from membranes. However, the latter process proceeds after the initial binding of PDC-109 to membranes with a lower time constant. Our results are of relevance for understanding the physiological role of PDC-109 binding to sperm cells. After ejaculation it is important that sperm cells do not perform the capacitation and acrosome reaction too early. Several factors have been discussed to stabilize sperm cells against a premature capacitation/acrosome reaction (see refs *43* and *44*). One of those factors is the high cholesterol content in the membrane of ejaculated sperm cells which rigidifies the membrane. With regard to our results another factor of cell stabilization might be the binding of proteins, e.g., seminal plasma proteins such as PDC-109, to the plasma membrane upon ejaculation, which results in a broad and effective immobilization of the plasma membrane.

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REFERENCES

1. Shivaji, S., Scheit, K.-H., and Bhargava, P. M. (1990) *Proteins of Seminal Plasma*, John Wiley and Sons, New York.
2. Cross, N. L. (1993) *Mol. Reprod. Dev. 35*, 316−323.
3. Bedford, J. M. (1983) *Biol. Reprod. 28*, 108−120.
4. Jones, R. (1990) *J. Reprod. Fertil. 42*, 89−105.
5. Bedford, J. M. (1994) *Hum. Reprod. 9*, 2187−2199.
6. Esch, F. S., Ling, N. C., Böhlen, P., Ying, S. Y., and Guillemin, R. (1983) *Biochem. Biophys. Res. Commun. 113*, 861−867.
7. Scheit, K. H., Kemme, M., Aumüller, G., Seitz, J., Hagendorff, G., and Zimmer, M. (1988) *Biosci. Rep. 8*, 589−608.
8. Calvete, J. J., Raida, M., Sanz, L., Wempe, F., Scheit, K. H., Romero, A., and Töpfer-Petersen, E. (1994) *FEBS Lett. 350*, 203−206.
9. Gerwig, G. J., Calvete, J. J., Töpfer-Petersen, E., and Vliegenthart, J. F. G. (1996) *FEBS Lett. 387*, 99−100.
10. Therien, I., Bleau, G., and Manjunath, P. (1995) *Biol. Reprod. 52*, 1372−1379.
11. Gasset, M., Saiz, J. L., Laynez, J., Sanz, L., Gentzel, M., Töpfer-Petersen, E., and Calvete, J. J. (1997) *Eur. J. Biochem. 250*, 735−744.
12. Gasset, M., Magdaleno, L., and Calvete, J. J. (2000) *Arch. Biochem. Biophys. 374*, 241−247.
13. Moreau, R., Frank, P. G., Perreault, C., Marcel, Y. L., and Manjunath, P. (1999) *Biochim. Biophys. Acta 1438*, 38−46.
14. Moreau, R., and Manjunath, P. (1999) *Biochim. Biophys. Acta 1438*, 175−184.
15. Müller, P., Erlemann, K. R., Müller, K., Calvete, J. J., Töpfer-Petersen, E., Marienfeld, K., and Herrmann, A. (1998) *Eur. Biophys. J. 27*, 33−41.
16. Constantine, K. L., Madrid, M., Bányai, L., Trexler, M., Patthy, L., and Llinás, M. (1992) *J. Mol. Biol. 223*, 281−298.
17. Desnoyers, L., and Manjunath, P. (1992) *J. Biol. Chem. 267*, 10149−10155.
18. Manjunath, P., Chandonnet, L., Leblond, E., and Desnoyers, L. (1994) *Biol. Reprod. 50*, 977.
19. Therien, I., Moreau, R., and Manjunath, P. (1998) *Biol. Reprod. 59*, 768−776.
20. Davis, B. K., Byrne, R., and Bedigian, K. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1546−1550.
21. Go, K. J., and Wolf, D. P. (1985) *Biol. Reprod. 32*, 145−153.

22. Ehrenwald, E., Foote, R. H., and Parks, J. E. (1990) *Mol. Reprod. Dev. 25*, 195−204.
23. Langlais, J., Kan, F. W. K., Granger, L., Raymond, L., Bleau, G., and Roberts, K. D. (1988) *Gamete Res. 20*, 185−201.
24. Marsh, D. (1995) *Mol. Membr. Biol. 12*, 59−64.
25. Marsh, D., and Horvath, L. I. (1998) *Biochim. Biophys. Acta 1376*, 267−296.
26. Fellmann, P., Zachowski, A., and Devaux, P. F. (1994) *Biomembrane Protocols: II. Architecture and Function* (Graham, J. M., and Higgins, J. A., Eds.) pp 161−175, Humana Press Inc., Totowa, NJ.
27. Calvete, J. J., Varela, P. F., Sanz, L., Romero, A., Mann, K., and Töpfer-Petersen, E. (1996) *Protein Expression Purif. 8*, 48−56.
28. Mayer, L. D., Hope, M. J., Cullis, R. P., and Janoff, A. S. (1985) *Biochim. Biophys. Acta 817*, 193−196.
29. Marx, U., Lassmann, G., Wimalasena, K., Müller, P., and Herrmann, A. (1997) *Biophys. J. 73*, 1645−1654.
30. Williams, R. M., Graham, J. K., and Hammerstedt, R. H. (1991) *Biol. Reprod. 44*, 1080−1091.
31. Parks, J. E., Arion, J. W., and Foote, R. H. (1987) *Biol. Reprod. 37*, 1249−1258.
32. Nikolopoulou, M., Soucek, D. A., and Vary, J. C. (1985) *Biochim. Biophys. Acta 815*, 486-498.
33. Parks, J. E., and Hammerstedt, R. H. (1985) *Biol. Reprod. 32*, 653−668.
34. Zalata, A. A., Christophe, A. B., Depuydt, C. E., Schoonjans, F., and Comhaire, F. H. (1998) *Mol. Hum. Reprod. 4*, 111−118.
35. Fattal, E., Nir, S., Parente, R. A., and Szoka, F. C., Jr. (1994) *Biochemistry 33*, 6721−6731.
36. Matsuzaki, K., Murase, O., Fujii, N., and Miyajima, K. (1996) *Biochemistry 35*, 11361−11368.
37. Müller, P., Schiller, S., Wieprecht, T., Dathe, M., and Herrmann, A. (2000) *Chem. Phys. Lipids 106*, 89−99.
38. Zachowski, A. (1993) *Biochem. J. 294*, 1−14.
39. Müller, K., Müller, P., and Herrmann, A. (1997) *J. Reprod. Fertil. 111*, 81−89.
40. Müller, K., Pomorski, T., Müller, P., and Herrmann, A. (1999) *J. Cell Sci. 112*, 11−20.
41. Müller, K., Pomorski, T., Müller, P., Zachowski, A., and Herrmann, A. (1994) *Biochemistry 33*, 9968−9974.
42. Calvez, J. Y., Zachowski, A., Herrmann, A., Morrot, G., and Devaux, P. F. (1988) *Biochemistry 27*, 5666−5670.
43. Nolan, J. P., and Hammerstedt, R. H. (1997) *FASEB J. 11*, 670−682.
44. Martínez, P., and Morros, A. (1996) *Front. Biosci. 1*, 103−117.

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