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ARTICLE

The bovine seminal plasma protein PDC-109 extracts phosphorylcholine-containing lipids from the outer membrane leaflet

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Abstract The bovine seminal plasma protein PDC-109 modulates the maturation of bull sperm cells by removing lipids, mainly phosphatidylcholine and cholesterol, from their cellular membrane. Here, we have characterized the process of extraction of endogenous phospholipids and of their respective analogues. By measuring the PDC-109-mediated release of fluorescent phospholipid analogues from lipid vesicles and from biological membranes (human erythrocytes, bovine epididymal sperm cells), we showed that PDC-109 extracts phospholipids with a phosphorylcholine headgroup mainly from the outer leaflet of these membranes. The ability of PDC-109 to extract endogenous phospholipids from epididymal sperm cells was followed by mass spectrometry, which allowed us to characterize the fatty acid pattern of the released lipids. From these cells, PDC-109 extracted phosphatidylcholine and sphingomyelin that contained an enrichment of mono- and di-unsaturated fatty acids as well as short-chain and lyso-phosphatidylcholine species. Based on the results, a model explaining the phospholipid specificity of PDC-109-mediated lipid release is presented.

Keywords Seminal plasma protein · PDC-109 · Phospholipid · Membrane

Abbreviations

BSA	Bovine serum albumin
HBS	Hepes buffered salt solution
L/P	Lipid to protein molar ratio
LUV	Large unilamellar vesicle(s)

Astrid Tannert and Anke Kurz have contributed equally to this work.

Dedicated to Prof. K. Arnold on the occasion of his 65th birthday.

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MALDI-TOF MS	Matrix-assisted laser desorption and ionization time-of-flight mass spectrometry
NBD-PC	1-Palmitoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino] caproyl]phosphatidylcholine
NBD-PE	1-Palmitoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]phosphatidylethanolamine
NBD-PL	NBD-labelled phospholipids
NBD-PS	1-palmitoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]phosphatidylserine
NBD-SM	6-[(*N*-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)amino]hexanoyl)sphingosylphosphatidylcholine
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
RBC	Red blood cells
SM	Sphingomyelin
TLC	Thin layer chromatography

Introduction

During their maturation, mammalian sperm cells undergo a complex series of molecular modifications that finally prepare the cells for fusion with the oocyte. These modifications are part of a poorly understood maturation process named capacitation, which includes an intense structural remodeling of the plasma membrane. During epididymal transit, the plasma membranes of sperm cells are subject to species-dependent changes of their lipid composition. In the case of bull sperm cells, the cholesterol/phospholipid ratio increases after removal of phospholipids (James et al. 1999) and the proportion of poly-unsaturated fatty acids is enhanced (Poulos et al. 1973). While sperm cells capacitate, certain lipids are laterally rearranged whereas others are removed from the plasma membrane (Gadella et al. 1995; Cross 1998; Travis and Knopf 2002). During the acrosome reaction, which follows capacitation and precedes fusion with the oocyte, the sperm membrane structure is remodeled in order to ensure the fusion between the plasma membrane and the outer acrosomal membrane (Nolan and Hammerstedt 1997; Allen and Green 1997).

Several components of mammalian seminal plasma are involved in the modulation of the membrane remodeling processes (Shivaji et al. 1990). Among these, proteins of the Fn type II family are of special importance. This protein family is characterized by domains (Fn type II) originally described in fibronectin (Skorstengaard et al. 1984). Bull seminal plasma contains various Fn type II proteins, which are designated as BSP-A1, BSP-A2, BSP-A3 and BSP-30kDa (collectively called BSP proteins) (Manjunath and Sairam 1987; Manjunath et al. 1987). These proteins are secretory products of the bovine seminal vesicles and constitute the major protein fraction of bovine seminal fluid. BSP-A1 and -A2, which are also termed PDC-109 (Esch et al. 1983), contain two tandemly arranged Fn type II domains. (Wah et al. 2002).

Early work from one of the authors provided evidence for a physiological role of PDC-109 during the maturation of bull sperm cells (Thérien et al. 1995; 1997). It was found that epididymal sperm cells, which are difficult to capacitate under in vitro conditions, undergo capacitation more rapidly in the presence of PDC-109. This physiological impact of PDC-109 seems to be associated with its capacity to remove lipids, mainly phosphatidylcholine (PC) and cholesterol, from the sperm membrane, thus changing the plasma membrane composition (Thérien et al. 1998, 1999; Moreau et al. 1999). The main lipids of bovine sperm cell membranes are PC, sphingomyelin (SM), phosphatidylethanolamine (PE) (50, 13, 10% of total phospholipid) and cholesterol (40% of total lipid) (Parks et al. 1987). It is very likely that the modulation of the lipid composition of sperm cells, particularly with regard to cholesterol, is a key process during sperm cell capacitation (Davis et al. 1980; Go and Wolf 1985; Langlais et al. 1988; Ehrenwald et al. 1990). This is underlined by the fact that the capacitation of sperm cells can be induced in vitro solely by incubating cells with methyl-ß-cyclodextrin (Choi and Toyoda 1998; Visconti et al. 1999; Iborra et al. 2000; Pommer et al. 2003), which is known to extract cholesterol from membranes (Yancey et al. 1996; Ohvo and Slotte 1996).

Although it has been shown that the PDC-109-mediated lipid extraction is different from that triggered by high-density lipoproteins (Thérien et al. 1998), the molecular mechanism underlying the extraction is not well understood. A selective extraction of PC from membranes can be rationalised since PDC-109 specifically interacts with this phospholipid (Desnoyers and Manjunath 1992; Müller et al. 1998; Ramakrishnan et al. 2001). However, the preference of PDC-109 for PC extraction could also be explained by a protein-mediated release of phospholipids solely from the outer membrane monolayer. Since PC is the main lipid of the outer membrane leaflet of bull spermatozoa (Nolan et al. 1995), a selective extraction of phospholipids from this leaflet would also result in a preferred release of PC by PDC-109. Moreover, several studies have demonstrated an interaction of PDC-109 with the outer leaflet of lipid membranes (Ramakrishnan et al. 2001; Greube

et al. 2001). On the other hand, PDC-109 may also influence the inner membrane leaflet as seen from its ability to disturb membrane integrity (Ramakrishnan et al. 2001; Greube et al. 2001; Gasset et al. 2000; A. Tannert and P. Müller, unpublished results). Two possibilities arise from these observations; (1) PDC-109 may extract phospholipids solely from the outer membrane leaflet and, if so, (2) the PDC-109-mediated release from this leaflet may exhibit lipid specificity.

In the present study, we have characterized the PDC-109-mediated phospholipid extraction from membranes with regard to (1) its leaflet specificity, i.e. whether phospholipids are released from both leaflets or solely from the outer membrane leaflet and (2) its lipid specificity, i.e. the headgroup(s) and fatty acid(s) of the released phospholipids. To verify this, we investigated the extraction of fluorescent phospholipids from lipid vesicles, human erythrocytes and caudal epididymal sperm cells. Moreover, we showed that PDC-109 could extract long chain phospholipids from lipid vesicles. The data related to the PDC-109-mediated phospholipid release were compared with those associated with the bovine serum albumin (BSA)-mediated extraction of lipid analogues from membranes. Special emphasis was given to the mass spectrometry analysis of the endogenous phospholipids released from caudal epididymal sperm cells by PDC-109. Our data indicate that PDC-109 extracts phopholipids mainly from the outer membrane leaflet of erythrocytes, sperm cells and lipid vesicles. With regard to lipid specificity, the protein preferentially triggers the release of phospholipids bearing a phosphorylcholine headgroup and having (1) acylated unsaturated fatty acids, (2) short chain fatty acids or (3) solely one fatty acid (lysoPC).

Materials and methods

Materials

Fluorescent phospholipids (NBD-PL), 1-palmitoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl] phosphatidylcholine (NBD-PC), 1-palmitoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]phosphatidylethanolamine (NBD-PE), 1-palmitoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]phosphatidylserine (NBD-PS), and 6-[(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl)sphingosylphosphatidylcholine (NBD-SM) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Lipids, egg yolk PC, egg yolk PE and egg yolk SM, bovine brain phosphatidylserine (PS), cholesterol, fatty acid free BSA and all other chemicals were from Sigma (Deisenhof, Germany).

HEPES buffered salt (HBS) solution contained 5 mM HEPES and 145 mM NaCl, pH 7.4. ATP-restoration buffer contained 60 mM NaCl, 40 mM KCl, 50 mM Na_2HPO_4, 10 mM sodium pyruvate, 10 mM adenosin, 10 mM inosine, 10 mM glucose, 1 mM $MgCl_2$.

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 (Calvete et al. 1996). Protein purity was verified by SDS-polyacrylamide gel electrophoresis, reverse-phase HPLC analysis and mass spectrometric analysis. For subsequent 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. PDC-109 was mixed with membranes (see below) at lipid to protein molar ratios (L/P) 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 protein bound to sperm cells (Calvete et al. 1994).

Preparation of multilamellar vesicles (MLV) and of large unilamellar vesicles (LUV)

Lipids were transferred from a chloroform stock solution into a glass tube. When applicable, fluorescent analogues were added at this step (final analogue concentration 1 mol%), allowing LUV to be labelled symmetrically, i.e. analogues were equally distributed on both membrane leaflets. The solvent was removed under a stream of nitrogen and lipids were resuspended in a small volume of ethanol [final ethanol concentration was below 1% (v/v)]. HBS was added and the mixture was vortexed to induce the formation of MLV having a final lipid concentration of 1–4 mM. For the preparation of LUV, the above suspension was subjected to five freeze-thaw-cycles and extruded through a 100 nm-diameter polycarbonate filter (Nucleopore GmbH, Tübingen, Germany) using either an extruder (Extruder, Lipex Biomembranes Inc., Vancouver, Canada) or a mini-extruder (Avanti Polar Lipids, Alabaster, AL, filters from Costar) (Mayer et al. 1985).

To prepare LUV exclusively labelled on the inner leaflet, fluorescence of lipid analogues located on the outer leaflet of symmetrically labelled LUV was destroyed by chemical reduction (Dao et al. 1991). Briefly, 360 µl of a 4 mM LUV solution, symmetrically labelled with NBD-PC or NBD-PS in HBS, were mixed with 40 µl of a sodium dithionite solution (100 mM Tris–HCl, 1 M sodium dithionite, pH 10) and incubated for 30 min on ice. To terminate the reduction,

dithionite was separated from LUV by gel filtration on a Sephadex column.

Interaction of proteins with NBD-PL-labelled LUV

To achieve a high time resolution, the kinetics of interaction between PDC-109 and LUV were measured using a stopped-flow approach at 37°C. Appropriate volumes of NBD-PL-labelled LUV and PDC-109 or BSA solution in HBS were loaded onto a stopped-flow device (RX 2000, Applied Photophysics, Leatherhead, UK) linked to an Aminco Bowman Series 2 spectrofluorometer (see below). The final lipid concentration of LUV was 50 μM with various amounts of protein. Fluorescence decrease due to interaction of proteins with lipid analogues in membranes was recorded over at least 150 s. Five single measurements were accumulated for each sample to increase the signal to noise ratio.

Interaction of proteins with MLV

Multilamellar vesicles consisting of PC were incubated with BSA or PDC-109 ($L/P = 20$) for 2 min at 37° and centrifuged (15 min, 15,000*g*). Phospholipids in the supernatant were recovered by organic extraction (Bligh and Dyer 1959) and the phospholipid content was quantified by measuring the phospholipid phosphorus after destruction of lipids with 70% perchloric acid for 30 min at 180°C (Rouser et al. 1966).

Preparation of human red blood cells (RBC)

Citrate-stabilized blood samples of healthy donors were purchased from the local blood bank (Berlin, Germany). RBC were washed twice with HBS (10 min, 2,000*g*) at 4°C. For treatment with *N*-ethylmaleimide (NEM), RBC were preincubated with 2 mM NEM for 15 min at 37°C and washed twice in HBS (10 min, 2,000*g*) at 4°C.

Extraction of NBD-PL from erythrocytes

The NBD-PL were transferred from a chloroform stock solution into a glass tube and the solvent was removed under a stream of nitrogen. Subsequently, the analogues were hydrated by adding HBS and vortexing. Washed erythrocytes were diluted with ATP-restoration buffer to a hematocrit of 25 (corresponding to a total lipid concentration of about 1.25 mM). For labelling the exoplasmatic leaflet of erythrocytes, one volume of the aqueous analogue solution was added to one volume of RBC suspension, incubated for 10 min at 4°C and washed twice with ice-cold HBS to remove non-incorporated analogues. The final analogue concentration was about 1 mol% of endogenous RBC phospholipids. To label the cytoplasmic leaflet of erythrocytes with NBD-PC, the analogue concentration was increased up to 5 mol%. To ensure the accumulation of the analogue in the inner leaflet, RBC were incubated for 14 h at 25°C (Wüstner et al. 1998). Analogues on the exoplasmatic leaflet were removed by incubating the cells twice with BSA [1% (w/v)]. Any residual BSA was removed by two washing steps with ice-cold HBS. Subsequently, these inner leaflet-labelled cells were mixed with PDC-109 or BSA (final $L/P = 20$). Cells and proteins were incubated at 25°C to slow down the transbilayer movement of analogues during the experiments, which could interfere with the measurement of extraction (see below). The protein-induced release of phospholipid analogues was determined by taking aliquots at different time intervals. The aliquots were centrifuged (45 s, 16,000*g*) and supernatant and pellet were carefully separated. Lipids from supernatant and pellet were extracted by the addition of 500 μl 2-propanol. After centrifugation (45 s, 16,000*g*), 400 μl of the supernatants were transferred to a fluorescence cuvette containing 1.6 ml 2-propanol and fluorescence intensities measured (see below). The extent of NBD-PL hydrolysis was measured upon lipid extraction by thin layer chromatography (TLC) and was found to be below 5% for all analogues within the experimental time course.

Preparation of caudal epididymal sperm cells

Bovine testes were purchased from the local slaughterhouse and the epididymides from two animals were dissected as previously described (Williams et al. 1991). Sperm cells from the cauda were obtained by fine mincing of epididymal tissue in a petri dish with Hank's solution (glucose supplemented, 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 Hank's solution. After a second centrifugation, cells were pooled and resuspended again in Hank's solution to give a final cell concentration of about 1.5×10^9 cells/ml.

Extraction of NBD-PC from epididymal sperm cells

Washed sperm cells were diluted with Hank's solution to a concentration of 0.75×10^9 cells/ml. This corresponds to a plasma membrane lipid concentration of

about 1.25 mM assuming a lipid content of 1.7 μmol for the plasma membrane of 10^9 sperm cells (Parks et al. 1987). Labelling and washing of cells with NBD-PC was carried out as described for RBC with the exception that all steps were performed at 25°C, in order to avoid the deterioration of caudal cells caused by low temperatures. The measurement of protein-induced analogue extraction was carried out as described for RBC.

Fluorescence measurements

All fluorescence spectra and kinetics were recorded using an Aminco Bowman spectrometer series 2 (SLM-Aminco, Rochester, NY, USA). NBD-labelled analogues were excited at 470 nm (slit width 4 nm) and fluorescence intensities were recorded at an emission wavelength of 540 nm (slit width 4 nm). Stopped-flow measurements were performed with a time resolution of 0.2 or 0.5 s.

Analysis of endogenous phospholipids extracted from caudal epididymal sperm cells by mass spectrometry

Caudal epididymal cells (0.75×10^9/ml) were incubated at 37°C in the absence or presence of PDC-109 ($L/P = 2$). After 30 min, samples were centrifuged (13,000*g*, 10 min) and lipids from the supernatants and pellets were isolated by organic extraction (Bligh and Dyer 1959; Schiller et al. 2003). In some cases, phospholipids were separated by TLC prior to mass spectrometry (Schiller et al. 2003). Phospholipids were analyzed by matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF) as described recently (Schiller et al. 2003). Briefly, a 20 μl aliquot of the organic extract of sperm cells (containing about 2.5 μg lipids) was mixed with 20 μl of the corresponding matrix solution (0.5 M 2, 5-dihydroxybenzoic acid), applied onto the sample plate and dried under a warm gas stream. All MALDI-TOF mass spectra were acquired on a Voyager Biospectrometry DE workstation (PerSeptive Biosystems, Framingham, MA, USA). The system utilizes a pulsed nitrogen laser emitting at 337 nm. The extraction voltage was 20 kV and the "low-mass gate" was turned on to prevent the saturation of the detector by ions resulting from the matrix (Petkovic et al. 2001). One hundred and twenty eight single laser shots were averaged for each mass spectrum. The laser strength was kept about ten percent above the threshold to obtain an optimum signal to noise ratio. In order to enhance the spectral resolution, all spectra were acquired in the reflector mode.

Results

Interaction of PDC-109 and BSA with NBD-PL symmetrically incorporated into LUV

To investigate the interaction of PDC-109 with phospholipids in membranes, LUV composed of PC or PC/cholesterol were symmetrically labelled with different fluorescent phospholipid analogues and the fluorescence was recorded upon addition of the protein. To better resolve the kinetics of the fluorescence change at 37°C, measurements were performed using the stopped-flow technique (see Materials and methods). Mixing of NBD-PL-labelled LUV with PDC-109 resulted in a decrease of fluorescence intensity (Fig. 1a–d). The kinetics of the fluorescence decrease were dependent on the analogue head-group, on the *L/P* ratio and on the presence or absence of cholesterol. Upon addition of PDC-109, the fluorescence decreased more rapidly in the case of LUV labelled with the choline-containing lipids NBD-PC and NBD-SM, compared to LUV labelled with NBD-PS and NBD-PE. A very slow fluorescence decline was observed for LUV labelled with NBD-PE. In all the PDC-109 experiments (Fig. 1a–d), the same order was maintained with respect to the extent of fluorescence decrease (NBD-PC > NBD-SM > NBD-PS > NBD-PE), with the exception of PC-LUV at the L/P ratio of 5, where the fluorescence decrease of NBD-PS-labelled LUV was greater than that of NBD-SM-labelled LUV (Fig. 1a). Although the decline of fluorescence was slower in the presence of cholesterol, we observed the same preference for interaction of PDC-109 with NBD-PC and NBD-SM. In the absence of PDC-109 we observed a very low decrease of fluorescence (Fig. 1a, b, only shown for NBD-PC in PC-LUV and in PC/cholesterol-LUV). These data indicate that PDC-109 is able to extract phospholipid analogues from lipid membranes and prefers choline-containing lipids to aminophospholipids. The interaction of PDC-109 with membranes does not interfere with bilayer integrity under our experimental conditions. Bilayer stability perturbation has only been observed for high PDC-109/lipid ratios and in the absence of cholesterol (Gasset et al. 2000; Ramakrishnan et al. 2001; Greube et al. 2001).

For comparison, we also followed the time-dependent decrease of fluorescence upon mixing BSA and labelled vesicles. BSA is known to extract NBD-PL from membranes, resulting in a fluorescence decrease due to a lower fluorescence quantum yield of analogues bound to BSA compared to those localized in the membrane (Colleau et al. 1991; Marx et al. 1997). In line with this, we observed a decrease of NBD fluorescence when BSA was added to labelled PC- or

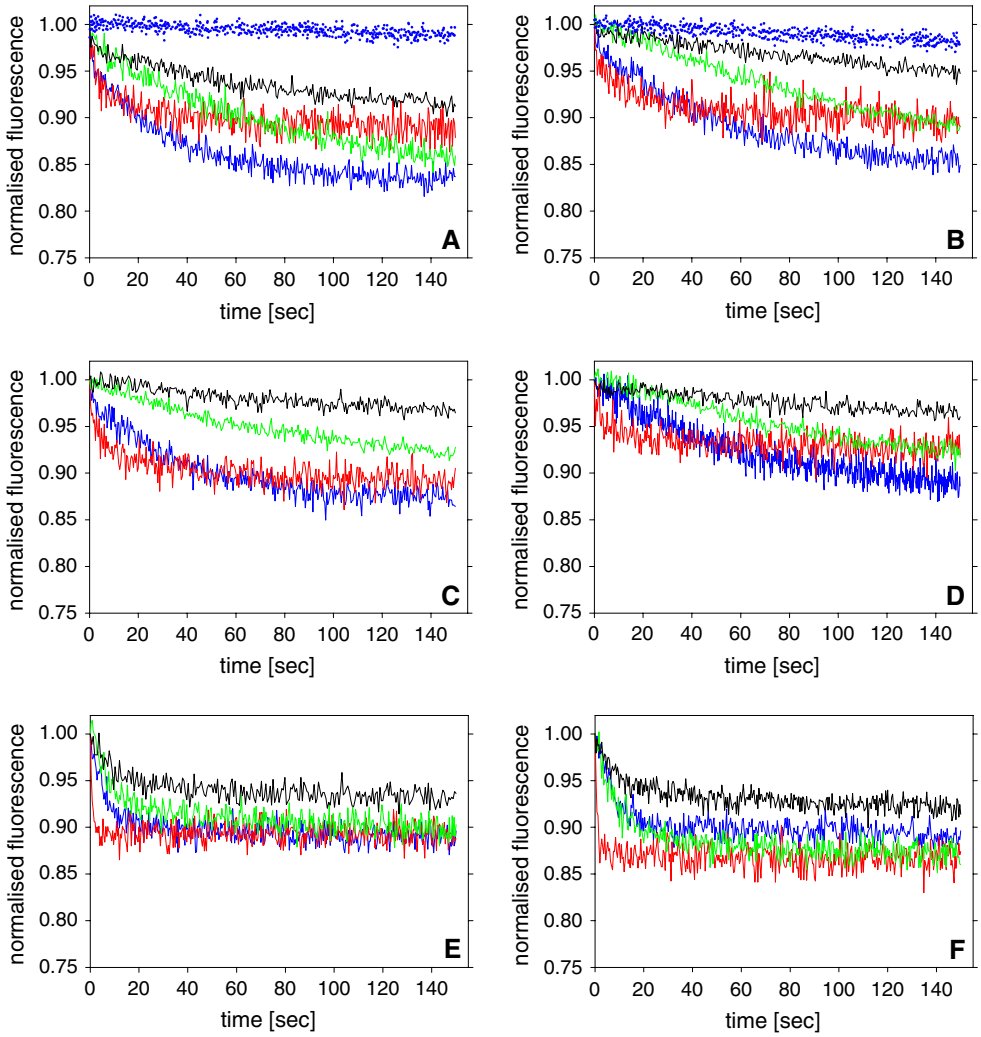

Fig. 1 Interaction of PDC-109 (**a**, **b**, **c**, **d**) and BSA (**e**, **f**) with NBD-PL-labelled LUV. LUV composed of PC (**a**, **c**, **e**) or PC and 30 mol% cholesterol (**b**, **d**, **f**) were labelled with NBD-PC (*blue lines*), NBD-SM (*red lines*), NBD-PS (*green lines*) or NBD-PE (*black lines*). LUV were mixed with PDC-109 or BSA in a stopped-flow fluorescence cuvette and fluorescence was measured at 37°C as described in Materials and methods. The final molar lipid to protein ratio was 5 (**a**, **b**, **e**, **f**) and 10 (**c**, **d**). The *blue dotted lines* in (**a**) and (**b**) show the fluorescence signal of NBD-PC in the absence of protein. *Each curve* represents the mean of five single measurements. *Each data set* shows the results from one representative experiment out of two independent measurements

PC/cholesterol-LUV (Fig. 1e, f, only shown for $L/P = 5$). The BSA-mediated changes of fluorescence were similar for PC and for PC/cholesterol liposomes. Comparing different phospholipid species, the fluorescence decrease upon addition of BSA was similar for NBD-PC and NBD-PS whereas it was somewhat slower for NBD-PE and faster for NBD-SM.

Interaction of PDC-109 with NBD-PL incorporated in the inner leaflet of LUV

We next investigated whether PDC-109 was able to extract lipid analogues from the inner leaflet of liposomes. To achieve this, LUV (composed of PC or PC/cholesterol) were labelled selectively on their inner leaflet with NBD-PC or NBD-PS. When PDC-109 was added to these LUV (measured up to $L/P = 5$), no changes in fluorescence intensity were observed within 10 min at 37°C (data not shown). Likewise, addition of BSA to vesicles labelled on the inner leaflet did not cause any changes in fluorescence under these conditions (data not shown). These results indicate that the interaction of PDC-109 with lipid membranes is confined to the outer leaflet, thereby causing a lipid extraction solely from this leaflet.

PDC-109-mediated extraction of phospholipids from MLV

The ability of PDC-109 to extract endogenous lipids from vesicles was measured by using MLV. These

vesicles can be easily centrifuged, allowing the separation of the supernatant from the membranes (Contreras et al. 2005). After incubation of PC-MLV with PDC-109 ($L/P = 20$) for 2 min at 37°C, 9.6 ± 4.9% ($n = 4$) of the total PC was found in the supernatant, indicating that PDC-109 extracted (long chain) phospholipids from the lipid vesicles. In contrast, almost no phospholipid was detected in the supernatant after incubation of vesicles with BSA (1.4 ± 2.8%; $n = 4$).

Interaction of PDC-109 with NBD-PL in erythrocytes

To investigate the ability of PDC-109 to extract lipids from a biological membrane, we employed human RBC. These cells were used as a model system since the leaflet-specific lipid composition of RBC membranes is well known. Moreover, since RBC lack internal membrane structures, the extent of labelling of the inner membrane leaflet is not affected by a labelling of intracellular compartments. As a result, the leaflet specificity of the PDC-109-membrane interaction can be easily characterized. In a first approach, the outer membrane leaflet of RBC was labelled with NBD-PL. Labelled erythrocytes were incubated with PDC-109 or BSA ($L/P = 20$) at 25°C, centrifuged and the amount of analogues in the pellet and supernatant was determined for 1 h. PDC-109 mediated an extraction of lipid analogues from the outer leaflet of the erythrocyte membrane (Fig. 2). The extent of released lipids was largest for the PC and SM analogues. For NBD-PS, we found a much lower amount of the analogue in the supernatant whereas extraction of NBD-PE was almost negligible. The half times of the PDC-109-mediated analogue (NBD-PC, NBD-SM, NBD-PS) release were in the range of 3–7 min. Pretreatment of RBC with NEM neither influenced the extraction of NBD-PC nor that of NBD-PS (data not shown).

As was already known, BSA extracted NBD-PL rapidly from the outer leaflet of erythrocyte membranes (Colleau et al. 1991; Fig. 2). The kinetics of extraction from the outer leaflet also showed a dependence on the phospholipid head group. The extent of released analogues decreased in the following order: NBD-PS > NBD-PC > NBD-SM = NBD-PE. Compared with PDC-109, the kinetics of BSA-mediated NBD-PC, NBD-SM and NBD-PS release were faster and could not be resolved with the centrifugation assay. The half time of BSA-mediated NBD-PE extraction was about 3 min.

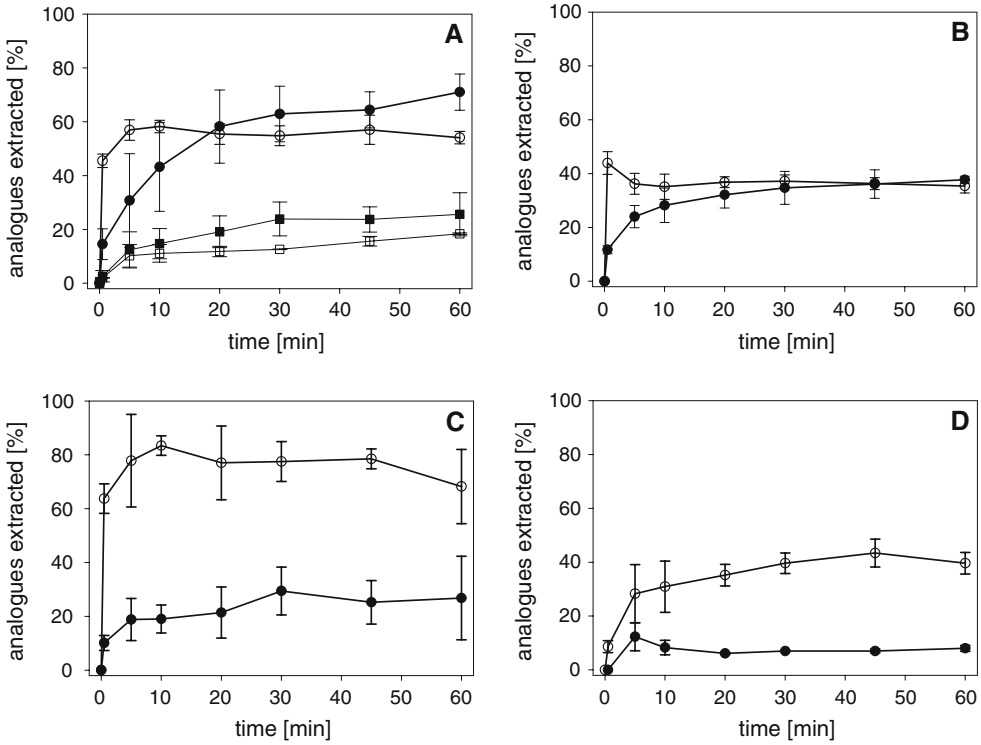

Fig. 2 Interaction of PDC-109 with NBD-PL-labelled human erythrocytes. Erythrocytes were labelled on the outer (*circles*) or on the inner (*squares*) leaflet with NBD-PC (**a**), NBD-SM (**b**), NBD-PS (**c**) or NBD-PE (**d**) as described in Materials and methods. Labelled cells were mixed with PDC-109 (*closed symbols*) or BSA (*open symbols*) at a *L/P* ratio of 20 and incubated at 25°C. At different time intervals, an aliquot of the suspension was taken, centrifuged and the amount of analogues in the pellet and supernatant was determined. Data represent the mean ± SD of at least three independent measurements

In a second approach, we specifically labelled the inner leaflet of erythrocytes with NBD-PC and investigated whether the analogue could also be extracted from this leaflet. To this end, we incubated inner leaflet-labelled RBC in the presence of PDC-109 or BSA ($L/P = 20$) (Fig. 2a). At $t = 0$, no analogues could be extracted by either PDC-109 or BSA, indicating that all analogues were localized in the cytoplasmic leaflet. However, after incubation for 60 min with PDC-109 or BSA, approximately 20% of NBD-PC was found in the supernatant. This amount of analogue is similar to that which has been reported to flip from the inner to the outer leaflet in RBC within 1 hour (Bitbol and Devaux 1988; Connor et al. 1992). Thus, PDC-109 and BSA extracted analogues from the exoplasmic leaflet that had been redistributed from the inner leaflet. These data indicate that PDC-109 mainly extracts lipids from the outer membrane leaflet of RBC.

Interaction of PDC-109 with NBD-PC in epididymal sperm cells

Next, we characterized the interaction of PDC-109 with caudal epididymal sperm cells. Since NBD-PS and NBD-PE rapidly translocate from the outer to the inner plasma membrane leaflet of mammalian sperm cells (Müller et al. 1994; Nolan et al. 1995), which interferes with the investigation of PDC-109-membrane interactions (see below), we solely employed the fluorescent analogue of PC.

In the first set of experiments, the outer membrane leaflet of sperm cells was labelled with NBD-PC and the release of the analogue was measured after addition of PDC-109 or BSA ($L/P = 20$) at 25°C. Upon addition of PDC-109 to labelled cells, we observed a rapid extraction of the analogue with a half time of about 1.5 min (Fig. 3a). BSA caused also a rapid extraction of NBD-PC, which was faster compared to PDC-109 and could not be resolved with the centrifugation assay (Fig. 3a). In a second set of experiments, we measured the protein-mediated release of NBD-PC from the inner membrane leaflet of caudal epididymal sperm cells (Fig. 3b). Immediately after addition of the proteins ($L/P = 20$), there were no analogues in the supernatant, indicating that all NBD-PC was localized in the cytoplasmic leaflet. As the incubation time increased, we observed an elevation in the amount of analogues extracted in the presence of PDC-109 and BSA. The kinetics of analogue extraction were similar for both proteins, but were significantly slower compared to those found for outer leaflet-labelled cells.

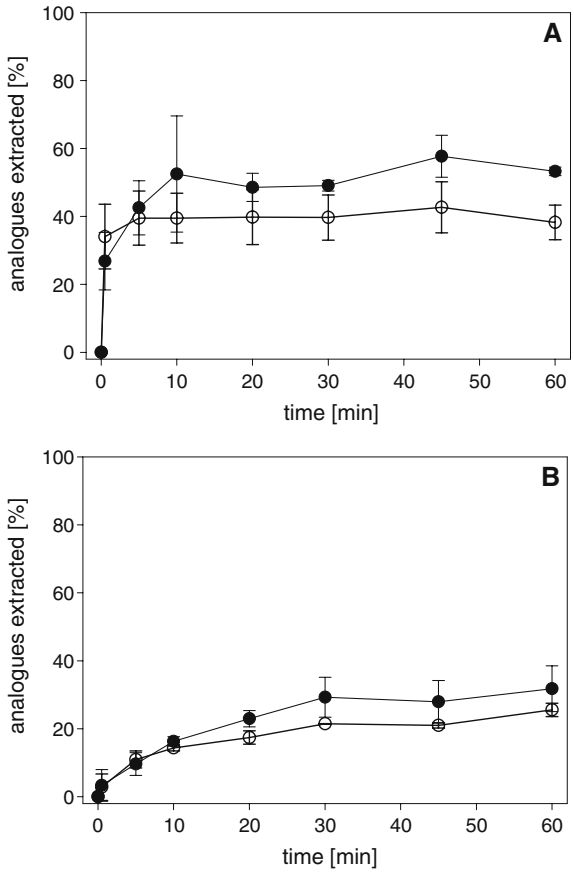

Fig. 3 Interaction of PDC-109 with NBD-PC-labelled epididymal sperm cells. Bovine epididymal sperm cells from the cauda were labelled on the outer (**a**) or on the inner (**b**) leaflet with NBD-PC as described in Materials and methods. Labelled cells were mixed with PDC-109 (*closed symbols*) or BSA (*open symbols*) at a *L/P* ratio of 20 and incubated at 25°C. At different time intervals, an aliquot of the suspension was taken, centrifuged and the amount of analogues in the pellet and supernatant was determined. Data represent the mean ± SD of two independent measurements

Analysis of endogenous phospholipids extracted from caudal epididymal sperm cells by PDC-109

Endogenous phospholipids extracted from epididymal sperm cells by PDC-109 were analyzed by MALDI-TOF MS. Peak intensities were related to the peaks of the matrix substance (DHB) which, under conditions of laser irradiation, gives rise to typical oligomerization products (e.g. a peak at $m/z = 551$) that can be used as an internal standard (Schiller et al. 2004).

The spectra obtained for the lipid composition of bovine caudal epididymal sperm cells were very similar to those obtained for ejaculated cells as measured recently by the same technique (Fig. 4a) (Schiller et al. 2003). When cells were incubated without protein and the supernatant was analyzed after centrifugation, very small peaks were observed compared to the intense

matrix peak at $m/z = 551$ (Fig. 4b) (Petkovic et al. 2001). After the incubation of caudal sperm cells with PDC-109 ($L/P = 2$) for 30 min at 37°C, significant peaks were detected in the supernatant. These peaks reflected a broad spectrum of lipids, mainly of the PC species (Fig. 4c, Table 1). Compared to the results obtained for control cells (incubated without protein), an increase in the relative intensity of several peaks in the supernatant of protein-incubated cells was observed, corresponding to (1) SM (16:0) and (2) unsaturated diacylated PC species (see Table 1). Moreover, PDC-109 extracted highly unsaturated alkyl–acyl and alkenyl–acyl PC species from caudal sperm cells (Fig. 4c, Table 1). To better differentiate the phospholipid species that were extracted by PDC-109, lipids from the supernatants of protein-incubated cells were separated by TLC and the spots were analyzed by MALDI-TOF MS (see Materials and methods). There were no spots corresponding to PE that could be detected on the TLC plates (not shown). This is unlikely to be due to a methodological problem since the approach is capable of detecting PE, as demonstrated for lipid extracts of whole cells (not shown, Schiller et al. 2003). The PC peaks observed in the MALDI-TOF MS spectra of protein-incubated cells (see Fig. 4c) were also found after TLC separation of lipids and exclusive analysis of the PC spot by mass spectrometry. Additionally, due to the higher sensitivity of the method, we identified additional PC species; PC 14:0/16:0 and PC 14:0/14:0. Analysis of the TLC spot representing SM and lysoPC by MALDI-TOF MS revealed that several peaks displayed significantly increased intensity compared to the control spectrum (supernatants of cells without PDC-109, compare Figs. 5a, b). The different lipid species assigned to these peaks are given in Table 1.

Discussion

In the present study, we characterized the process of phospholipid extraction mediated by the bovine seminal plasma protein PDC-109. We showed that PDC-109

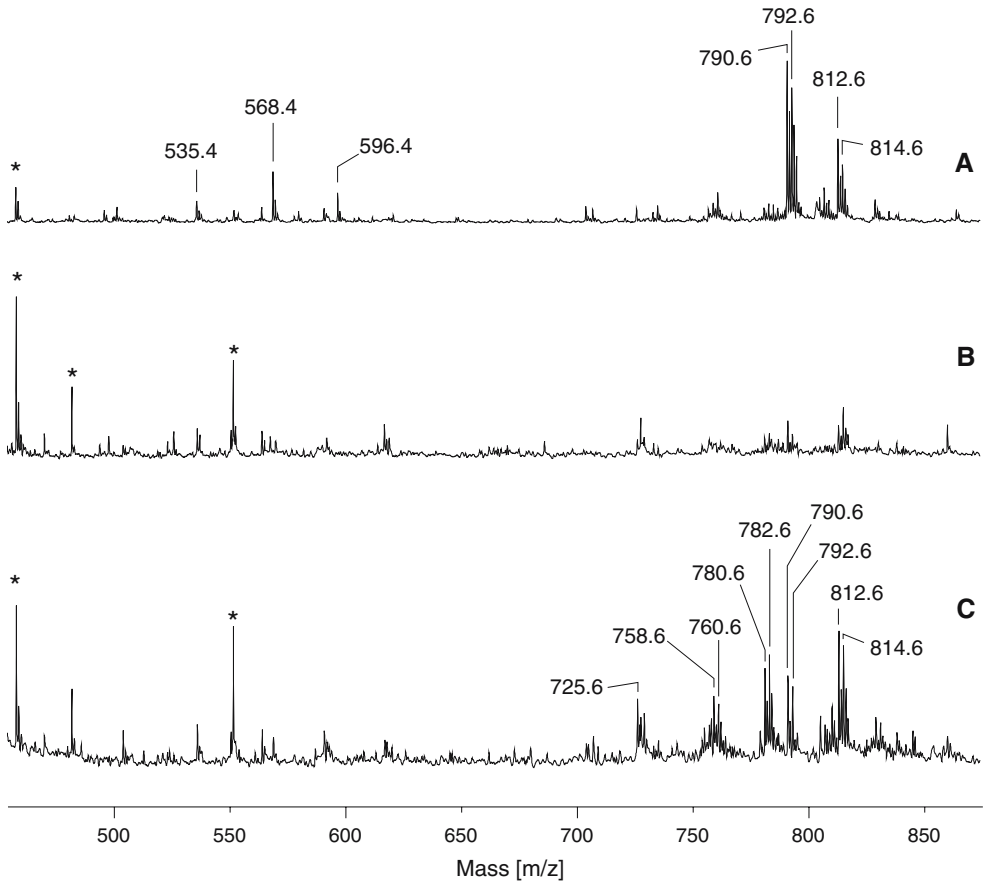

Fig. 4 Endogenous phospholipids extracted from bovine epididymal sperm cells by PDC-109. Positive ion MALDI-TOF mass spectra were recorded from organic extracts of caudal sperm cells (**a**) and from the supernatant of caudal cells incubated in the absence (**b**) and in the presence (**c**) of PDC-109 ($L/P = 2$) for 30 min at 37°C. Peaks are labelled according to their m/z ratios and the asterisks represent characteristic matrix peaks. All spectra were scaled with reference to the most intense peak. The different amounts of phospholipids in the individual fractions are visible when the intensity of the matrix peaks (e.g. $m/z = 551$) is used for comparison

Table 1 Overview of the observed *m/z* values in the positive ion MALDI-TOF mass spectra of organic extracts of PDC-109-incubated caudal sperm cells and their corresponding assignment to individual phospholipid species

Peak position	Assignment of molecular mass
Without separation[a]	
725.6	SM 16:0 (Na^+)[b]
758.6	PC 16:0; 18:2 (H^+)[b]
760.6	PC 16:0; 18:1 (H^+)[b]
780.6	PC 16:0; 18:2 (Na^+)[b]
782.6	PC 16:0; 18:1 (Na^+)[b]
790.6	PC 18:0; 18:0 (H^+) or PC 16:0; 22:6 (H^+) alkenyl–acyl
792.6	PE 18:0; 22:6 (H^+) or PC 16:0; 22:6 (H^+) alkyl–acyl
812.6	PC 18:0; 18:0 (Na^+) or PC 16:0; 22:6 (Na^+) alkenyl–acyl
814.6	PE 18:0; 22:6 (Na^+) or PC 16:0; 22:6 (Na^+) alkyl–acyl
LPC/SM fraction[c]	
480.3	Alkenyl LPC 16:0 (H^+)
482.3	Alkyl LPC 16:0 (H^+)
494.3	LPC 16:1 (H^+)
496.3	LPC 16:0 (H^+)
502.3	Alkenyl LPC 16:0 (Na^+)
504.3	Alkyl LPC 16:0 (Na^+)
516.3	LPC 16:1 (Na^+)
518.3	LPC 16:0 (Na^+)
568.3	LPC 22:6 (H^+)
590.3	LPC 22:6 (Na^+)
664	Impurity[d]
686	Impurity[d]
703.6	SM 16:0 (H^+)
725.6	SM 16:0 (Na^+)

[a] Caudal epididymal sperm cells were incubated with PDC-109 (see Materials and methods). Lipids in the supernatant were extracted and analysed by MALDI-TOF mass spectrometry

[b] Peaks that had a comparatively higher intensity with regard to those observed in spectra obtained from control cells not incubated with PDC-109

[c] Lipids of the supernatant from PDC-109-incubated caudal cells (see above) were separated by TLC. The spot containing the lysoPC and SM fraction was scraped off, eluted and analysed by MALDI-TOF MS

[d] See Klein et al. (1998)

(1) mediates a release of fluorescent phospholipid analogues from the outer membrane leaflet of lipid vesicles as well as of biological membranes, (2) extracts long chain PC from lipid membranes, (3) extracts endogenous phospholipids from epididymal bull sperm cells and (4) extracts phospholipids dependent on their headgroup from both model and biological membranes.

Although the extraction of endogenous lipids from several biological membranes (fibroblasts, RBC, sperm cells) by PDC-109 has been documented (Thérien et al. 1998, 1999; Moreau et al. 1999; Greube et al. 2004), this has not, to date, been reported for pure lipid membranes. Therefore, we also investigated the interaction of PDC-109 with lipid vesicles. We showed that PDC-109 is also capable of extracting phospholipids with two long fatty acid chains from liposomes. Since PDC-109 extracts short chain phospholipid analogues as well as long chain phospholipids from lipid vesicles, this indicates that the extraction of lipids from biological membranes does not require any interaction of PDC-109 with membrane proteins or with specific membrane receptors.

In order to characterize the PDC-109-mediated extraction of phospholipids from membranes (Thérien et al. 1998, 1999; Moreau et al. 1999), we employed fluorescent lipid analogues. The advantages of using NBD-PL for this purpose are (1) a rapid incorporation into membranes, (2) the possibility to specifically label the outer or inner membrane leaflet, (3) a strong dependence of the quantum yield on the local NBD environment and (4) the ability to investigate the lipid specificity of the extraction process. Although these analogues have a short fatty acid chain and an artificial NBD moiety, they have been successfully employed to investigate the role of lipids for various biological processes, such as transbilayer movement of lipids, lipid-protein interactions, lipid-bile salt interactions and intracellular transport processes of lipids (Wüstner et al. 1998; Pomorski et al. 1994; Wüstner et al. 2001; Kol et al. 2003; Im et al. 2004; Eckford and Sharom 2005). The qualitatively similar results obtained with regard to the lipid-specific interaction of PDC-109 with NBD-PL (this study) and with endogenous and spin-labelled phospholipids (Desnoyers and Manjunath 1992; Müller et al. 1998; Ramakrishnan et al. 2001) supports the applicability of lipid analogues for characterizing PDC-109-mediated lipid extraction. In addition, the similar results obtained underline that the effect of the NBD moiety on the binding of labelled lipids to PDC-109 is minimal or non-existent.

When mixing PDC-109 and outer leaflet-labelled LUV, we observed a time-dependent decrease of fluorescence. These changes in fluorescence indicate a transfer of analogues from the liposomal membrane to the protein, similar to what was observed for BSA. Due to differences in the environment of the NBD moiety, analogues bound to the proteins have a lower quantum yield compared to those embedded in the membrane, explaining the fluorescence decrease. Comparison of the kinetics showed that analogues of PC and SM are extracted from LUV by PDC-109 at a faster rate than PS and PE analogues. In the presence of cholesterol, extraction of analogues by PDC-109 was slower compared to pure PC membranes. This can be explained by (1) a stronger anchorage of lipids to

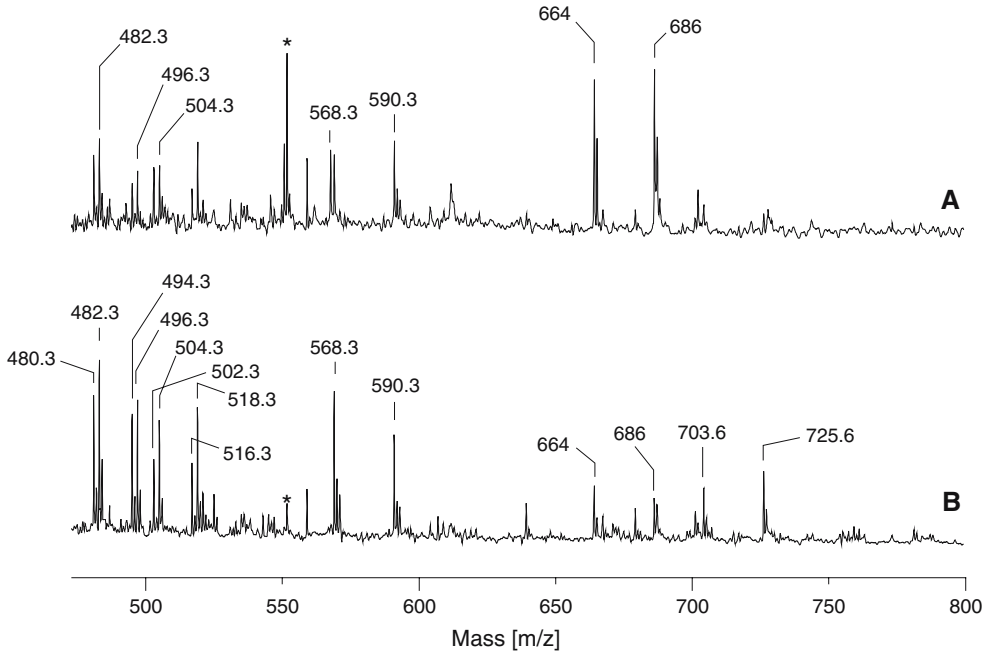

Fig. 5 Identification of phospholipid species extracted from bovine epididymal sperm cells by PDC-109. Caudal sperm cells were incubated in the absence (**a**) and in the presence (**b**) of PDC-109 ($L/P = 2$) for 30 min at 37°C. Lipids in the supernatant were isolated by organic extraction and separated by TLC in the absence of acids. The spots representing lysoPC and sphingomyelin were scraped off the TLC plate, eluted and analysed by positive ion MALDI-TOF MS. The peak marked with an *asterisk* was induced by the matrix. All spectra were scaled with reference to the most intense peak. The different amounts of phospholipids in the individual fractions are visible when the intensity of the matrix peak ($m/z = 551$) is used for comparison

cholesterol containing membranes due to tighter lipid packing (Davies et al. 1990; Huster et al. 1998), (2) a reduced affinity of PDC-109 for cholesterol-containing membranes and/or (3) a competition of cholesterol and PC for the protein's lipid binding sites (Swamy et al. 2002). The order of affinity observed for PDC-109-lipid interactions with regard to the NBD-PL head group was the same as found for spin-labelled PL (Ramakrishnan et al. 2001).

The PDC-109 also extracts endogenous phospholipids (Greube et al. 2004) and lipid analogues (this study) from human erythrocytes. The specificity of extraction of fluorescent lipids observed for RBC was similar to that found for liposomes. Upon addition of PDC-109, the amount of analogues in the supernatant increased more rapidly and to a higher extent for the choline-containing lipids than for the aminophospholipids. When investigating the extraction of lipid analogues from the outer membrane leaflet, it is possible that the amount of analogues in this leaflet could be reduced due to a transbilayer movement of the analogues to the inner leaflet, thus decreasing the effective concentration of the analogue in the outer leaflet. However, NBD-PC, SM and PE move across the RBC membrane very slowly by passive diffusion (Colleau et al. 1991), therefore any interference in the measure of the extraction of these analogues due to transbilayer movement can be neglected. For NBD-PS, transbilayer movement has to be considered since this lipid is transported from the outer to the inner membrane leaflet by the aminophospholipid translocase (Colleau et al. 1991; Connor et al. 1992). However, the inward movement of NBD-PS in RBC (Colleau et al. 1991; Pomorski et al. 1994, 1996) is slower than the PDC-109-mediated extraction of this analogue (Fig. 2). In order to completely exclude an influence of NBD-PS inward transport on the extraction kinetics, we have preincubated RBC with NEM, which is known to inhibit the aminophospholipid translocase (Zachowski et al. 1986). The kinetics of PDC-109-mediated extraction of NBD-PS from RBC pretreated with NEM was very similar to that in the absence of NEM. Therefore, the low level of NBD-PS release from RBC membranes induced by PDC-109 could not be explained by a decreasing analogue concentration in the outer leaflet due to lipid translocation. Although it has been described that PDC-109 has no affinity for PS (Desnoyers and Manjunath 1992; Müller et al. 1998; Ramakrishnan et al. 2001) we found a (low) protein-induced release of NBD-PS from RBC or from lipid vesicles. This can be explained by some affinity of the protein to short-chain phospholipids (see below) or by a concomitant, but to

a lower extent release of NBD-PS with endogenous PC (Thérien et al. 1999).

The PDC-109 and BSA extracted NBD-PC from the outer leaflet of caudal epididymal sperm cells to similar extents as observed for RBC. However, PDC-109 extracted the analogue from sperm cells more rapidly than from erythrocytes, which may be due to differences in the lipid composition of RBC and sperm cells. These differences could influence the physical state of the membrane and, thus, protein-mediated extraction. For example, sperm cells contain a large amount of highly unsaturated fatty acids. Recently, we have shown that the physical state of lipid membranes modulates their interaction with PDC-109, which interacts more effectively with highly fluid membranes (Greube et al. 2001).

The preferred extraction of PC from fibroblasts and bull epididymal sperm cells by PDC-109 was shown recently using radioactively labelled phospholipids (Thérien et al. 1998; 1999; Moreau et al. 1999). However, due to the high degree of PC labelling in comparison to other phospholipids present in the plasma membrane of sperm cells, it might be argued that the extraction of other phospholipids could have been underestimated in these conditions. Therefore, we employed MALDI-TOF MS to analyze the endogenous phospholipids extracted from caudal epididymal bull sperm cells by PDC-109. MALDI-TOF spectra of the supernatant of sperm cells incubated with PDC-109 revealed the presence of only the SM and PC species. The main non-choline-containing phospholipid present in bull sperm cells, PE, was not extracted. The fatty acid pattern of the SM and PC species, which were released from epididymal sperm cells in the presence of PDC-109, revealed an enrichment of (1) lysolipids, (2) unsaturated, and (3) short-chain lipids. These data could be explained, on one hand, by a preferred interaction of PDC-109 with these lipid species. In agreement with this, Thomas et al. (2003) and Anbazhagan and Swamy (2005) measured the association constants of PDC-109 with different lipid species, showing that the protein exhibits higher affinity for both lysoPC and diacyl PC than for diacyl phosphatidylglycerol, diacyl phosphatidic acid and diacyl PE. On the other hand, the extractability of lipids may depend on the hydration of their head group (Binder et al. 1999) and on their hydrophobicity, which is mainly determined by the length and the degree of saturation of the fatty acid residues (Heikinheimo and Somerharju 1998). According to the latter point, phospholipids with shorter and/or unsaturated fatty acids can be more easily released from membranes, as is observed in the presence of bile salts (Cohen et al. 1990; Wüstner et al. 1998). A third possible explanation, as we have shown here, involves the distribution of lipids in the outer and inner membrane leaflets (see Zachowski 1993). An enrichment of certain lipids in the outer membrane leaflet coupled with an extraction solely from this leaflet would explain the preferential removal of these lipid species by PDC-109.

For RBC and epididymal sperm cells, several lines of evidence indicate a preferential PDC-109-mediated extraction of lipids from the outer membrane leaflet. First, the amount of NBD-PC extracted was significantly higher for outer leaflet-labelled cells than for inner leaflet-labelled cells. Second, the rates for the PDC-109-mediated extraction of NBD-PC were different for the outer and inner leaflet-labelled cells. To express these differences in a quantitative manner, we fitted the data to a single exponential equation and calculated the initial velocities of analogue extraction (v_i). The calculated values were 7.5% analogue/min and 2.5% analogue/min for the outer and the inner leaflets of RBC, respectively, and 75.9% analogue/min and 2.3% analogue/min for the outer and the inner leaflets of epididymal sperm cells, respectively. We are aware that for the very rapid kinetics of analogue release, v_i values may only reflect a lower limit of extraction rate. Third, while the kinetics of extraction from the outer leaflet were different for BSA and PDC-109, they are similar for both proteins with respect to the inner monolayer.

The detection of a minor NBD-PC release from inner leaflet-labelled cells in the presence of PDC-109 or BSA reflects the fraction of analogue molecules redistributing to the outer membrane leaflet during the time course of the experiment, where they became available for extraction. NBD-PC moves from the inner to the outer leaflet of RBC by slow passive diffusion (Bitbol and Devaux 1988; Connor et al. 1992). Thus, for inner leaflet-labelled cells, the rate-limiting step is the redistribution of analogues from the inner to the outer leaflet and not the protein-mediated extraction from the outer leaflet.

In summary, two mechanisms ensure that PDC-109 exclusively extracts choline-containing phospholipids from sperm cell membranes. One concerns the preferential interaction of PDC-109 with these lipids (Desnoyers and Manjunath 1992; Müller et al. 1998; Ramakrishnan et al. 2001) and the other is based on the selective release of phospholipids from the outer membrane leaflet and an asymmetric transbilayer phospholipid distribution. Mammalian sperm cells mainly contain PC and SM in their outer plasma membrane leaflet, whereas PS and PE are concentrated on the inner leaflet (Hinkovska et al. 1986; Müller et al.

1994; Nolan et al. 1995). Our data contribute to understand the molecular process of phospholipid extraction mediated by PDC-109.

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