

# Transbilayer motion of spin-labelled phospholipids in the plasma membrane of epididymal and ejaculated ram spermatozoa

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The transbilayer movement and distribution of spin-labelled phospholipid analogues were studied in the plasma membrane of ram sperm cells isolated from functionally different regions of the epididymis (caput, cauda) and from the ejaculate. As already shown for ejaculated cells, (i) a rapid movement of aminophospholipid analogues phosphatidylserine and phosphatidylethanolamine from the exoplasmic to the cytoplasmic leaflet, and (ii) a slow transbilayer movement of phosphatidylcholine were observed in the plasma membrane of maturing ram sperm cells. This suggests an asymmetric steady state transbilayer distribution of phospholipids, with a preferential enrichment of aminophospholipids on the cytoplasmic leaflet of those cells. The fast inward redistribution of the aminophospholipid analogues is consistent with the presence of an aminophospholipid translocase activity in all three sperm cell preparations. The translocase activity was enhanced slightly during the epididymal transit of spermatozoa. However, compared with epididymal sperm cells, a marked increase in the aminophospholipid translocase activity and a more pronounced phospholipid asymmetry for phosphatidylethanolamine was established for ejaculated spermatozoa. The physiological relevance of the rapid removal of aminophospholipids from the exoplasmic leaflet of the plasma membrane of ram sperm cells is discussed. The quality of sperm cell fractions was characterized by the cell morphology, membrane integrity and the cellular ATP concentration.

## Introduction

Epididymal transit and ejaculation are crucial events in mammalian spermatogenesis. During epididymal maturation, functional changes such as acquisition of motility and potential ability to penetrate the egg have been reported (Amann, 1987; Williams *et al.*, 1991; Hinton *et al.*, 1996). The contact with seminal fluid triggers further preparative events which prime the spermatozoa for fertilization. Therefore, the plasma membrane of sperm cells acts as an essential target for various physiological stimuli that may evoke distinct alterations in its structure and properties. For example, several lines of evidence suggest that the lateral and transverse arrangement of lipids is important for the regulation of sperm cell function and may be specific for each maturation step (Bearer and Friend, 1982; Hammerstedt and Parks, 1985; Langlais and Roberts, 1985; Arts *et al.*, 1993; Benoff *et al.*, 1993; Wolf, 1994; Gadella *et al.*, 1995).

The movement of phospholipids between the outer and inner leaflet of the cell membrane of ejaculated ram spermatozoa is a highly dynamic, ATP-dependent process (Müller *et al.*, 1994a). Fluorescent and spin-labelled phospholipid analogues

were used to detect distinct lipid-specific mechanisms of transbilayer movement and an asymmetric phospholipid distribution between the two halves of the cell membrane, as have been shown to exist in a number of other eukaryotic biological membranes (Op den Kamp, 1979; Seigneuret and Devaux, 1984; Zachowski *et al.*, 1986; Martin and Pagano, 1987; Zachowski, 1993). A carrier protein, known as aminophospholipid translocase, specifically mediates a rapid ATP-dependent translocation of aminophospholipids from the outer to the inner leaflet with a half-time of < 2 min at 20°C for the spin-labelled analogues of phosphatidylserine and phosphatidylethanolamine (SL-PS and SL-PE) (Müller *et al.*, 1994a). Phosphatidylserine (PS) and phosphatidylethanolamine (PE) are enriched in the cytoplasmic membrane leaflet; the choline lipids, phosphatidylcholine (PC) and sphingomyelin (SM), are enriched in the outer one (Müller *et al.*, 1994a). The ATP-dependent transport of aminophospholipids in the ram sperm cell membrane is very efficient since, only after a marked reduction of the cellular ATP concentration to about 5%, the fast inward translocation but not the asymmetric steady state distribution of PS and PE was significantly decreased (Müller *et al.*, 1994a). A similar picture of transbilayer movement and distribution of phospholipids emerged from investigations of the plasma membrane of bull spermatozoa using fluorescent analogues (Nolan *et al.*, 1995). It is presumed that such an

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arrangement and the dynamics of phospholipids between plasma membrane leaflets are not unique to mammalian sperm cells, since qualitatively similar characteristics for trout spermatozoa have been observed (Müller *et al.*, 1994b).

The efficiency of the ATP-dependent translocation of aminophospholipids in ejaculated sperm cells implies that this process and the maintenance of the asymmetric transbilayer distribution of phospholipids in the plasma membrane may be essential for sperm cells not only after ejaculation but perhaps even at earlier stages. Therefore, the present study investigated the transbilayer movement and distribution of spin-labelled phospholipid analogues SL-PC, SL-PE and SL-PS in ram sperm cells isolated from the morphologically separate epididymal regions, the cauda and the caput, respectively, as well as in ejaculated ram sperm cells. The analogues bearing a short-chain fatty acid with the NO-moiety in the  $\beta$ -position are incorporated readily from the external solution into the exoplasmic leaflet of plasma membranes and mimic quite faithfully the behaviour of endogenous phospholipids in biological membranes (Morrot *et al.*, 1989; Pomorski *et al.*, 1996). Their transbilayer redistribution was assessed by back exchange of spin-labelled analogues remaining in the outer leaflet to BSA (Calvez *et al.*, 1988; Morrot *et al.*, 1989). This 'back-exchange assay' allows a fast and gentle treatment of sperm cells (Müller *et al.*, 1994a).

## Materials and Methods

### Preparation of spermatozoa

Ejaculated spermatozoa from 2–5-year-old Merino, Blackhead and Texel rams, which continuously give semen throughout the year, were washed twice in a modified Beltsville thawing solution (mBTS) containing 205 mmol glucose  $l^{-1}$ , 20 mmol sodium citrate  $l^{-1}$ , 15 mmol  $NaHCO_3$   $l^{-1}$ , 10 mmol  $KCl$   $l^{-1}$ , 1.1 mmol  $MgCl_2$   $l^{-1}$  and 1 mmol EGTA  $l^{-1}$  (pH 7.4) and were treated as described by Müller *et al.* (1994a). Ram epididymal spermatozoa from the same animals were prepared within 2 h after slaughter. For each experiment, the epididymis from one or two animals was dissected according to the method described by Williams *et al.* (1991). Spermatozoa from caput and cauda were obtained by fine mincing of epididymal tissue in a Petri dish with Hank's solution supplemented with glucose (without calcium and magnesium) and subsequent filtration through paper tissues. Spermatozoa were then centrifuged at 800 g for 10 min, and the pellet was resuspended in 20 volumes of mBTS. After a second centrifugation, cells were pooled and resuspended in mBTS again to about  $1.5$  or  $2.5 \times 10^9$  cells  $ml^{-1}$  for caput and cauda sperm cells, respectively.

### Labelling of ram sperm cells

If not stated otherwise, all steps were performed at room temperature. Spin-labelled phospholipids, 1-palmitoyl-2-(4-doxy-pentanoyl)-phosphatidylcholine (SL-PC), -phosphatidylethanolamine (SL-PE) and -phosphatidylserine (SL-PS), were synthesized as described by Fellmann *et al.* (1994) and dissolved in chloroform:methanol (1:1). Analogues were

transferred to a glass tube, dried under nitrogen and vortexed with the desired volume of mBTS before addition to the cell suspension.

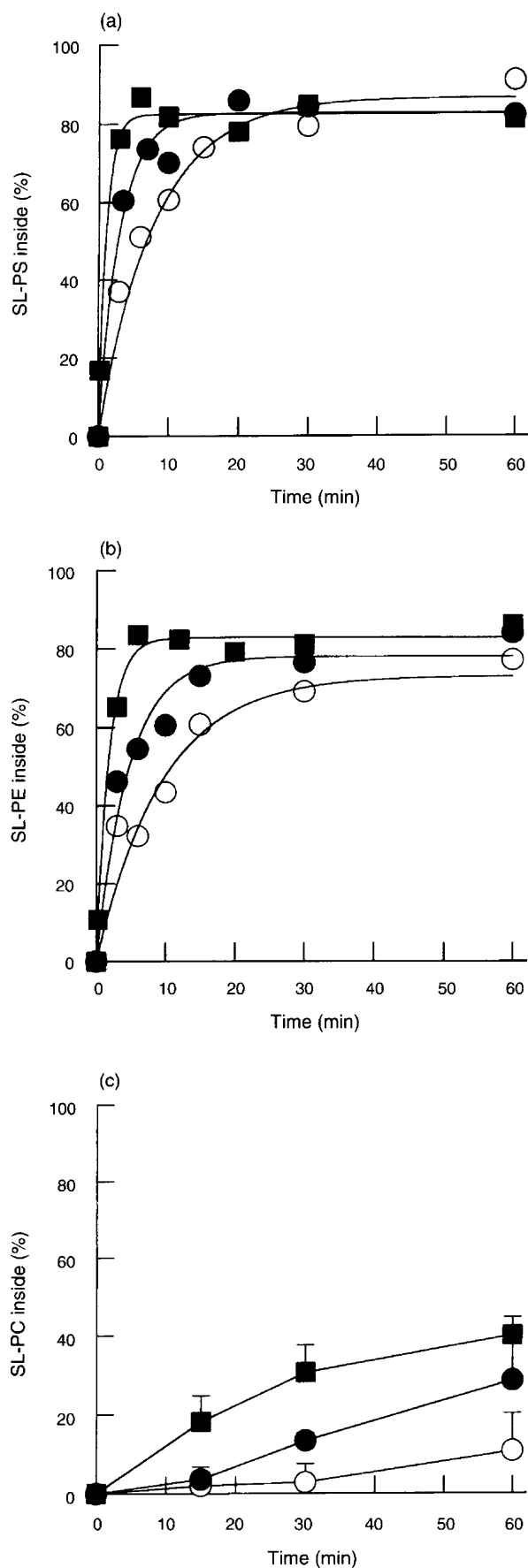
Hydrolysis of labelled phospholipids was prevented by preincubating three volumes of cells with one volume of di-isopropylfluorophosphate (DFP, Aldrich, Steinheim) in mBTS, prepared from a stock solution, to give a final concentration of 5 mmol  $l^{-1}$  (Müller *et al.*, 1994a). After equilibration for 5 min, two volumes of DFP containing cell suspension were mixed with one volume of label suspension representing time 0 for all kinetic measurements. The label concentrations were 3.6%, 2.1% and 1.8% of the concentration of endogenous membrane phospholipids for caput, cauda and ejaculated sperm cells, respectively. Owing to experimental constraints, namely the sensitivity of ESR equipment and the limited available number of cells, a larger amount of label was applied to caput sperm cells. Total uptake of the spin-labelled analogues into membranes occurs within 15 s after probe addition (Müller *et al.*, 1994a).

### Back-exchange assay for spin-labelled phospholipid analogues

For back exchange, 140  $\mu l$  samples were transferred at given time points to 60  $\mu l$  3% fatty acid-free BSA (Sigma, Deisenhofen) in mBTS on ice and centrifuged after incubation for 1 min at 12 000 g for 30 s. More than 95% of spin-labelled lipid analogues in the outer membrane leaflet can be extracted by BSA under these conditions (Müller *et al.*, 1994a). The amount of probe present in each BSA-supernatant was estimated from its electron spin resonance (ESR) spectrum intensity after reoxidation by ferricyanide (10 mmol  $l^{-1}$ ) using a Bruker ECS 106 (Karlsruhe, Germany) or a Miniscope MS100 (Magnetech, Berlin) spectrometer. Owing to the rapid and significant redistribution of aminophospholipids to the inner leaflet in ejaculated ram spermatozoa even within the short time period of about 30 s between labelling of sperm cells and performing the first back exchange, these translocation kinetics were fitted to time 0 to assess the amount of analogues initially incorporated into the outer leaflet (Müller *et al.*, 1994a).

### Assessment of label hydrolysis

Even in the presence of DFP, hydrolysing activity was observed in ram spermatozoa cleaving the short chain spin-labelled fatty acid in the  $\beta$ -position of the analogues. The amount of hydrolysed label in the BSA supernatant can be quantified from the ESR spectrum. The difference between the ESR line shapes of the unrestricted tumbling spin-labelled fatty acids and the rather immobilized movement of spin-labelled phospholipid analogues in the ESR spectrum of the BSA extract allows a quantification of hydrolysis. All kinetics were corrected for hydrolysis according to Morrot *et al.* (1989). The extent of hydrolysis was documented as the percentage of the total amount of incorporated label. Label hydrolysis may affect the estimated transbilayer distribution of analogues. However, if hydrolysis does not exceed 15% of total label, the error of the estimated transverse distribution is within the accuracy of the back-exchange assay (T. Pomorski, P. Müller, J. Libera and



A. Herrmann, unpublished). A stronger hydrolysis would misrepresent a higher portion of label in the inner membrane leaflet.

#### Assessment of sperm cell quality

Each preparation of epididymal spermatozoa was observed under a microscope for sperm motility and tested for the number of sperm cells with cytoplasmic droplets, loose heads and contaminating blood cells. The normal acrosomal ridge of an intact acrosome was characterized by phase-contrast optics at a magnification of  $\times 1000$ . Simultaneously, differentiation of cell status was performed by fluorescence microscopy. Sperm cells were stained with the fluorescent dye, Hoechst 33258, which rapidly penetrates defective cells. Briefly,  $5 \mu\text{l}$  samples of sperm cells were diluted five times with mBTS and subsequently fixed with 2% Formol in mBTS (1/1, v/v). After dilution with mBTS, cells were stained with Hoechst 33258 as described by De Leeuw *et al.* (1991) and wet slides were prepared. The determination of cellular ATP content was performed on a BioOrbit Luminometer using an ATP monitoring kit (Colora, Lorch).

#### Statistical analysis

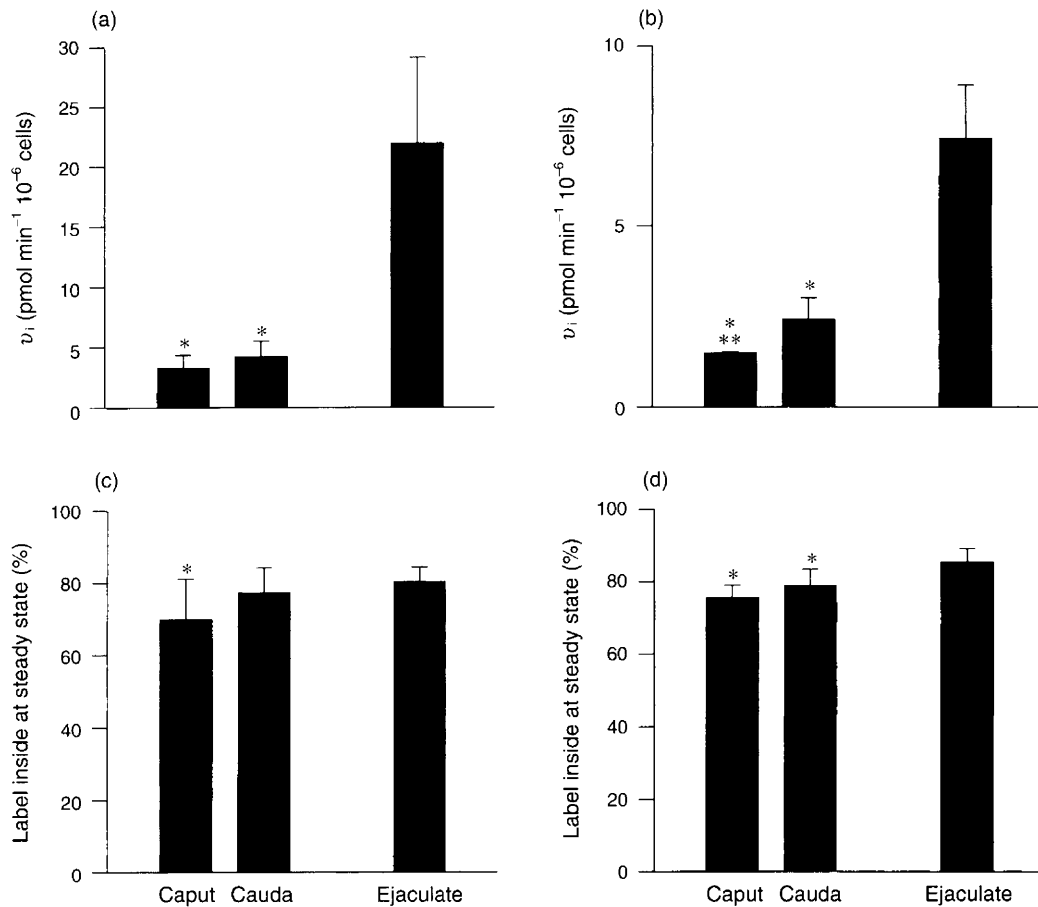
Data for the fast redistribution of aminophospholipid analogues were fitted to a monoexponential function (SigmaPlot). Results are expressed as mean  $\pm$  SD, with  $n$  being the number of experiments. Differences were analysed by Student's  $t$  test.

## Results

#### Transbilayer movement of spin-labelled phospholipid analogues in spermatozoa from epididymis and after ejaculation

The kinetics of the transbilayer redistribution of SL-PS and SL-PE, respectively, in the cell membrane of ram sperm cells from the caput epididymis, from the cauda epididymis and from the ejaculate at  $20^\circ\text{C}$  are shown (Fig. 1). Independent of the origin of sperm cells, the kinetics resemble qualitatively those reported for ejaculated spermatozoa by Müller *et al.* (1994a). The analogues SL-PS and SL-PE move rapidly to the cytoplasmic half of the plasma membrane, where they accumulate. The rapid inward redistribution of the spin-labelled

**Fig. 1.** Transbilayer redistribution of the spin-labelled phospholipid analogues (SL) of (a) phosphatidylserine (PS); (b) phosphatidylethanolamine (PE) and (c) phosphatidylcholine (PC) in the plasma membrane of epididymal and ejaculated ram sperm cells at  $20^\circ\text{C}$ . Spermatozoa from (○) caput epididymis, (●) cauda epididymis and (■) ejaculated ram sperm cells. At time  $t = 0$  the outer leaflet was labelled with the respective analogue. The fraction of label reorientated to the cytoplasmic side was determined by back-exchange assay. (a, b) Data of one characteristic experiment were fitted to a monoexponential function. (c) The numbers of experiments were four and seven for epididymal and ejaculated spermatozoa, respectively. Values are means  $\pm$  SD.



**Fig. 2.** (a, b) Initial velocity of transbilayer redistribution and (c, d) steady state distribution of the spin-labelled analogues of (a, c) phosphatidylserine (PS), and (b, d) phosphatidylethanolamine (PE) in epididymal and ejaculated ram sperm cells at 20°C. The initial velocity,  $v_i$  was obtained by monoexponential fitting of redistribution kinetics. Note the different scale of the ordinates for the initial velocity of translocation. The numbers of experiments were four and seven in the epididymal and ejaculated spermatozoa, respectively. Values are means  $\pm$  SD. \*Significantly different from ejaculated sperm cells ( $P < 0.01$  for (a) and (b) and  $P < 0.05$  for (c) and (d)); \*\*significantly different from caudal sperm cells ( $P < 0.05$ )

aminophospholipid analogues is not related to the reporter group, the NO moiety, since the same behaviour has been observed for phospholipid analogues bearing a fluorescent group completely different chemically from that of the spin label (Müller *et al.*, 1994a).

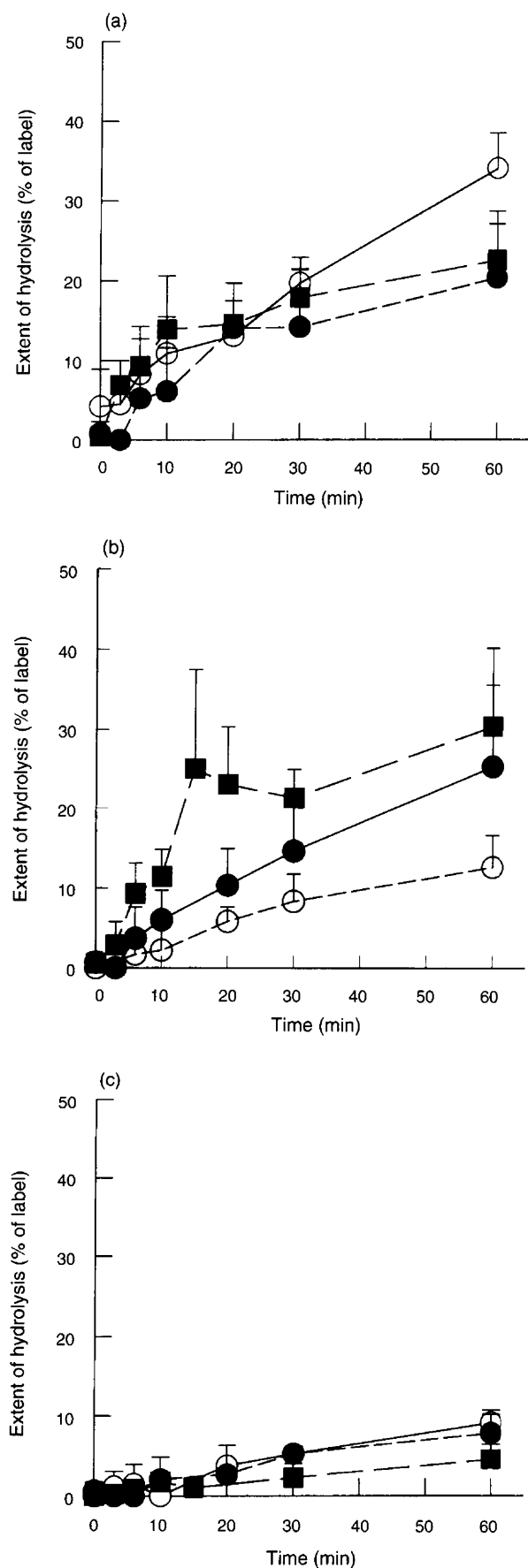
Significant quantitative differences in the translocation kinetics of both analogues were observed between ejaculated sperm cells and those from the epididymal regions. The transbilayer redistribution of aminophospholipids was much more rapid in ejaculated spermatozoa than in caput or cauda sperm cells (Fig. 1). For comparison of absolute values, the initial velocities of aminophospholipid translocation are given (Fig. 2). (Note the higher amount of label in caput sperm cells (see Discussion).) These were significantly lower in sperm cells from both epididymal parts compared with ejaculated ram sperm cells (see also, Müller *et al.*, 1994a). A slight but significantly slower inward translocation of SL-PS and SL-PE was observed in the plasma membrane of caput spermatozoa compared with sperm cells from the cauda (Figs 1 and 2).

In all three types of sperm cells, translocation of SL-PS was found to be 2–3 times faster than that of SL-PE (Fig. 2).

However, this difference in translocation between both aminophospholipid analogues is small in comparison with that observed for human red blood cells, which is in the order of about one degree of magnitude faster (Müller *et al.*, 1994a; Pomorski *et al.*, 1996).

In all ram sperm cells studied there was a pronounced asymmetric steady state transverse distribution of aminophospholipids with  $\geq 70\%$  of incorporated analogues on the cytoplasmic side. There was no significant deviation between the steady state transbilayer distribution of the two aminophospholipid labels for a given type of sperm cell (caput, cauda or ejaculate; Fig. 2). Similarly, the difference in the steady state transbilayer aminophospholipid distribution between sperm cells from caput and cauda epididymis was not significant (Fig. 2). However, the enrichment of SL-PS and SL-PE on the cytoplasmic side at steady state was more pronounced for ejaculated spermatozoa.

The transbilayer movement of the spin-labelled analogue of PC, SL-PC, differs clearly from that of the aminophospholipid analogues. Within 60 min after incorporation of label, only  $10.8 \pm 9.5$  and  $28.6 \pm 9.0\%$  of SL-PC moved to the cytoplasmic



membrane half at 20°C in caput and cauda sperm cells, respectively (Fig. 1). Internalization of SL-PC to the inner membrane leaflet was more efficient in cauda than in caput spermatozoa. However, even in ejaculated sperm cells, in which the rate and the extent of redistribution was higher than for epididymal spermatozoa, only  $40.0 \pm 4.6\%$  of total analogues were confined to the inner leaflet after 60 min.

#### *Extent of label hydrolysis in epididymal and ejaculated spermatozoa*

It is presumed that spin-labelled analogues are hydrolysed by the action of phospholipase-A<sub>2</sub> activity (see Roldan and Fragio, 1993). The ESR spectrum of the BSA extract allows a quantification of hydrolysis. After labelling of sperm cells, an increasing amount of spin-labelled analogues was hydrolysed despite the presence of DFP (see Fig. 3). The extent of this hydrolysis of SL-PS was similar for epididymal and ejaculated sperm cells, although it was greater for caput sperm cells at 60 min. Hydrolysis of SL-PE was greater in cauda than in caput sperm cells and greater still in ejaculated sperm cells. In caput spermatozoa, degradation of SL-PS was much greater than that of SL-PE. In cauda and ejaculated cells, both analogues were hydrolysed to a similar extent. The level of hydrolysis of SL-PC was relatively low compared with that of amino-phospholipids (< 10% within 60 min) and similar for all three types of spermatozoa investigated.

#### *Characterization of spermatozoa*

Morphological characteristics of the spermatozoa prepared from the two different regions of epididymis are shown (Table 1). Fifty-three per cent of the cells from the caput region had a cytoplasmic droplet proximal to the sperm head as a sign of immaturity. As expected, only 36% of cauda sperm cells had a cytoplasmic droplet, which had drifted to the more distal region of the sperm tail. In both cases, the number of loose heads was low ( $\leq 6\%$ ). In the caput fraction, contamination with blood cells reached 15% because of the dense blood capillary network in that region of the epididymis. Whereas caput sperm cells were nearly immotile, most cauda sperm cells showed a slow local movement. These characteristics infer the successful preparation of ram spermatozoa deriving from the two different regions of the epididymis. Ejaculated semen samples had > 80% motile cells and had few cytoplasmic droplets, loose heads or non-sperm cells.

#### *ATP content and quality of sperm cells*

The ATP content at the time of labelling was slightly, but significantly, lower for caput sperm cells compared with cauda

**Fig. 3.** Extent of di-isopropylfluorophosphate (DFP)-resistant hydrolysis of the spin-labelled phospholipid analogues of (a) phosphatidylserine (PS); (b) phosphatidylethanolamine (PE) and (c) phosphatidylcholine (PC) in epididymal and ejaculated ram sperm cells at 20°C. Spermatozoa from (○) caput epididymis, (●) cauda epididymis and (■) ejaculated ram sperm cells. The fraction of hydrolysed label is related to the total amount of incorporated label. The numbers of experiments were four and seven in the case of epididymal and ejaculated spermatozoa, respectively. Values are means  $\pm$  SD.

**Table 1.** The relative number (%)<sup>a</sup> of different cell types in two preparations of epididymal (caput and cauda) ram spermatozoa

	n	Sperm cells		Non-sperm cells	
		With cytoplasmic droplet <sup>b</sup>	Loose heads <sup>b</sup>	Erythrocytes	Leukocytes
Caput epididymis	4	78.8 ± 9.4			
		53.2 ± 22.5	6.0 ± 5.0	15.0 ± 4.1	5.6 ± 5.6
Cauda epididymis	4	95.3 ± 4.4			
		36.5 ± 21.2	4.2 ± 2.0	4.2 ± 3.2	0.3 ± 0.5

Values are means ± SD. Samples were taken from preparations used for spin labelling.

<sup>a</sup>Normalized to the total number of cells in the sample (sperm cells plus non-sperm cells).

<sup>b</sup>Normalized to the number of sperm cells in the sample.

**Table 2.** ATP content of epididymal (caput and cauda) and ejaculated ram spermatozoa at various times after incorporation of the spin-labelled phospholipid analogues, phosphatidylserine (SL-PS) and phosphatidylcholine (SL-PC)

	n	ATP content (nmol per 10 <sup>8</sup> sperm cells)	
		5 min	60 min
Caput epididymis			
SL-PS	4	11.7 ± 5.6	9.6 ± 7.3
SL-PC	4	9.7 ± 3.6	8.4 ± 5.3
Cauda epididymis			
SL-PS	4	15.3 ± 1.7	11.6 ± 1.0 <sup>c</sup>
SL-PC	4	16.1 ± 1.5 <sup>a</sup>	12.5 ± 0.8 <sup>c</sup>
Ejaculate			
SL-PS	7	15.6 ± 5.9 <sup>b</sup>	7.0 ± 2.8 <sup>c</sup>
SL-PC	5	12.6 ± 4.8	10.1 ± 2.3

Values are means ± SD.

<sup>a</sup>Significantly different from caput sperm cells with the same label ( $P < 0.01$ ).

<sup>b</sup>Significantly different from caput sperm cells with the same label ( $P < 0.05$ ).

<sup>c</sup>Significantly different from values obtained 5 min after labelling of sperm cells ( $P < 0.01$ ).

or ejaculated sperm cells (Table 2). Sixty minutes after incorporation of label, the ATP content was reduced in all sperm cell fractions. The most evident reduction, to 45% of the initial ATP concentration, was recorded for ejaculated cells labelled with SL-PS. Further studies are needed to elucidate whether the addition of SL-PS into the outer leaflet stimulates the aminophospholipid translocase and, thus, leads to an enhanced ATP consumption.

After preparation, spermatozoa from epididymis and ejaculates had a large proportion of cells with an intact acrosomal ridge unaffected by labelling (Table 3). While this proportion remained almost constant over the course of the experiment for caput and ejaculated cells, respectively, a significant loss of acrosomes was observed for cauda sperm cells. The same tendency was indicated by the penetration of the fluorescent dye H33258, which was used as an independent indicator for membrane disturbance. Cauda sperm cells were more

**Table 3.** Morphological parameters of epididymal (caput and cauda) and ejaculated ram spermatozoa at various times after incorporation of spin-labelled phospholipid analogues, phosphatidylserine (SL-PS) and phosphatidylcholine (SL-PC)

	n	NAR* (%)		Hoechst-negative and NAR <sup>†</sup> (%)	
		5 min	60 min	5 min	60 min
Caput epididymis					
SL-PS	4	81 ± 3	79 ± 5	75 ± 1	69 ± 4 <sup>d</sup>
SL-PC	4	84 ± 3	81 ± 7	73 ± 4	78 ± 6 <sup>a</sup>
Cauda epididymis					
SL-PS	4	76 ± 7	66 ± 2 <sup>a,d</sup>	56 ± 7 <sup>a</sup>	49 ± 2 <sup>a,c</sup>
SL-PC	4	75 ± 5 <sup>a</sup>	65 ± 10 <sup>a,e</sup>	56 ± 5 <sup>a</sup>	48 ± 3 <sup>a,d</sup>
Ejaculate					
SL-PS	5	84 ± 7 <sup>c</sup>	84 ± 4 <sup>b,c</sup>	nd	76 ± 5 <sup>a,c</sup>
SL-PC	5	86 ± 4 <sup>c</sup>	86 ± 6 <sup>c</sup>	nd	80 ± 13 <sup>c</sup>

Values are means ± SD. nd, not determined.

\*NAR, percentage of sperm cells with a normal acrosomal ridge (phase-contrast microscopy × 1000).

<sup>†</sup>Hoechst-negative and NAR: percentage of sperm cells with a normal acrosomal ridge not stained by Hoechst 33258.

<sup>a</sup>Significantly different from caput sperm cells with the same label ( $P < 0.01$ ).

<sup>b</sup>Significantly different from caput sperm cells with the same label ( $P < 0.05$ ).

<sup>c</sup>Significantly different from cauda sperm cells with the same label ( $P < 0.01$ ).

<sup>d</sup>Significantly different from values obtained 5 min after labelling of sperm cells ( $P < 0.01$ ).

<sup>e</sup>Significantly different from values obtained 5 min after labelling of sperm cells ( $P < 0.05$ ).

susceptible to the Hoechst dye H33258 (Table 3). Available data indicate that the connection between head and tail allows the entrance of the dye (K. Müller, P. Müller and A. Herrmann, unpublished; see also De Leeuw *et al.*, 1991). The matured cauda sperm cell, and particularly the integrity of the neck region, still lacking the protection of seminal plasma, seems to be very sensitive to experimental treatment. Since most ejaculated spermatozoa were impermeable to the dye, and the ATP content of cauda sperm cell samples was similar to that of spermatozoa after ejaculation, it was assumed that cauda sperm cells *in situ* were as intact as caput or ejaculated spermatozoa.

## Discussion

The transverse movement and distribution of phospholipids in the plasma membrane of ram sperm cells at different stages of their maturation were investigated for the first time by isolating and enriching them from separate, functionally different regions of the epididymis, the caput and the cauda, and after ejaculation. The transit of sperm cells through the epididymis and their subsequent ejaculation are accompanied by significant alterations in preparation for fertilization (see Amann, 1987; Hammerstedt and Parks, 1987; Hinton *et al.*, 1996). An important conclusion from the present study is that aminophospholipid translocase activity and asymmetric transbilayer phospholipid distribution are fundamental properties of the plasma membrane of epididymal as well as of ejaculated ram sperm cells.

A transmembrane movement of the four phospholipid analogues was found in epididymal sperm cells that qualitatively resembled that in fresh ejaculated ram sperm cells (see also Müller *et al.*, 1994a): SL-PS and SL-PE moved rapidly to the cytoplasmic leaflet whereas the analogues that contained choline disappeared slowly from the exoplasmic half. The fast reorientation of aminophospholipid analogues to the cytoplasmic side in epididymal spermatozoa is consistent with the presence of aminophospholipid translocase activity. Since the fast, unidirectional, ATP-dependent translocation of PS and PE has been shown to be essentially involved in maintaining the transverse phospholipid asymmetry in human erythrocyte membranes (Herrmann and Müller, 1986; Calvez *et al.*, 1988; Kuypers *et al.*, 1993; Brumen *et al.*, 1993; Heinrich *et al.*, 1997), it is assumed that a similar transbilayer distribution, with an enrichment of the aminophospholipids in the inner leaflet and of the choline phospholipids in the exoplasmic half of the plasma membrane, occurs in ram sperm cells during their maturation. Differential disappearance of labelled analogues gives no direct measure of an asymmetric steady state distribution between the outer and inner monolayer (Müller *et al.*, 1994a; Pomorski *et al.*, 1996); for example, exchange of probe between the cytoplasmic half of the plasma membrane and intracellular membranes may cause continuous removal of labelled lipids from the plasma membrane and, hence, the measurement of steady state distribution becomes impossible. Indeed, the steady state distribution of the phospholipid analogues in the plasma membrane of the various sperm cell fractions is similar to asymmetric transbilayer arrangements in other eukaryotic cells (for review, see Zachowski, 1993).

The aminophospholipid translocation is significantly increased in ejaculated sperm cells compared with epididymal sperm cells. The following reasons can be precluded as responsible for the observed difference:

First, the initial velocity of aminophospholipid translocation in ram sperm cells depends on the amount of analogue incorporated into the membrane and follows a saturation function (Müller *et al.*, 1994a). Owing to experimental constraints, more label (3.8%) was applied in caput compared with cauda and ejaculated sperm cells (about 2%). If the extent of labelling was responsible for the differences between the initial velocity of translocation of aminophospholipid analogues, the greatest velocity would have been expected for caput sperm cells. However, the opposite was the case. Hence, the low

efficiency of aminophospholipid translocation in the epididymal sperm cells, in particular those of the caput epididymis, cannot be an artefact of the higher concentration of incorporated label.

Second, spin-labelled lipid analogues are hydrolysed in ram sperm cells despite the presence of the phospholipase A<sub>2</sub>-inhibiting substance DFP. Hydrolysis of analogues might result in an incorrect estimation of the kinetics of transverse redistribution of analogues depending not only on the degree but also on the localization (in the exoplasmic or the cytoplasmic leaflet of the plasma membrane, or in both) of hydrolysis (T. Pomorski, P. Müller, J. Libera and A. Herrmann, unpublished). (Assuming there is no lipid specificity, hydrolysis in sperm cells may be localized on the cytoplasmic side of the cell, since the degree of label destruction coincides with the inward movement of analogues (T. Pomorski, P. Müller, J. Libera and A. Herrmann, unpublished). SL-PC, which displayed a slow transmembrane passage, was less degraded than the aminophospholipid analogues, which reoriented rapidly to the inner half.) As long as the extent of hydrolysis does not exceed 15%, the error of estimating the initial velocity of inward movement as well as the steady state transbilayer distribution of aminophospholipid analogues is in the order of about 5% despite the topology of hydrolysis. More importantly, the difference in the transmembrane passage of SL-PS between epididymal and fresh spermatozoa is evident only within the first 10 min after labelling but the degree of label hydrolysis in this period is similar and below 15%. In the case of SL-PE, analogue destruction gradually increased with the amount of cell maturation. However, the extent of hydrolysis was again below 15% within the first 10 min of the experiments for all ram sperm cell types investigated. Therefore, the hydrolysis of the analogues cannot account for the differences of inward movement established.

Third, it is unlikely that damage of the plasma membrane is responsible for the reduced initial velocity of aminophospholipid translocation in epididymal cells compared with ejaculated cells. Although a reduced normal acrosomal ridge and an enhanced penetration of fluorescent H33258 indicate membrane disturbance in cauda spermatozoa, there was no indication of a difference between the membrane integrity of caput and ejaculated sperm cells. Hence, the difference between the initial velocities of aminophospholipid translocation between the two latter fractions cannot be explained by membrane damage.

Fourth, fractions of caput sperm cells are contaminated by blood cells corresponding to about 15% of total cells. In this case, the initial velocity of aminophospholipid translocation is underestimated since, for example, in ram red blood cells this parameter is 1–2 orders lower than that of ejaculated ram sperm cells (Müller *et al.*, 1996). However, even assuming the rather extreme case that in 15% of isolated cells from the caput no translocation of aminophospholipids occurs, the true initial velocity would be higher by a factor of about 1.2. Since the difference in initial velocity between ejaculated sperm cells and cells from the caput corresponds to a factor of six, the contamination of caput spermatozoa fractions by blood cells cannot account for it.

Therefore, it is suggested that the increased inward motion of aminophospholipids, especially after ejaculation, is related to destined alterations of ram sperm cells, although, at this stage

of investigation, the origin of the increased aminophospholipid translocase activity is not known.

The cellular ATP concentration must be considered. A maturation-dependent change of the cellular ATP concentration could cause differences in the initial translocation velocity. However, from a previous study (Müller *et al.*, 1994a), it is surmised that even caput epididymal sperm cells have a sufficient ATP concentration to ensure a rapid aminophospholipid translocation comparable with that of ejaculated cells. In fresh ram sperm cells, only a drastic reduction of cellular ATP by a factor of 20, to 0.6 nmol ATP  $10^{-8}$  cells after inhibition of respiration and glycolysis could affect the initial velocity of SL-PS translocation without an impact on the steady state distribution. When only glycolysis was inhibited in these fresh sperm cells, the cellular ATP was reduced to about 2.9 nmol ATP  $10^{-8}$  cells and the redistribution of spin-labelled phospholipids remained unchanged (Müller *et al.*, 1994a). Since an ATP content similar to that of ejaculated spermatozoa was found in cauda sperm cells, a variation of cellular ATP concentration cannot be responsible for differences in the translocation kinetics between caput, cauda and ejaculate sperm cell preparations.

Enhancement of the ATP-dependent transport of aminophospholipids by stimulation of aminophospholipid translocase activity must also be considered. While the regulation of the translocase is still an open question, known modifications of the sperm plasma membrane upon ejaculation may be responsible for such a stimulus. It has been shown, using human erythrocytes, that aminophospholipid translocase activity becomes enhanced upon increasing the membrane fluidity by cholesterol depletion (Morrot *et al.*, 1989). Hammerstedt and Parks (1987) observed an increase in the fluidity of isolated ram sperm cell membrane fractions during epididymal transit and an accompanying increase in the amount of unsaturated fatty acids in membrane phospholipids. Wolf and Voglmayr (1984) also observed an enhancement of ram sperm cell plasma membrane fluidity, except in the midpiece region, after ejaculation.

Although it is beyond the scope of this study, it is possible that the increased aminophospholipid transport efficiency after ejaculation is of physiological relevance. A reasonable hypothesis is that the enhanced activity is required to avoid any additional appearance of aminophospholipids in the outer leaflet caused by disturbance of the plasma membrane and increased transverse passive movement of phospholipids after ejaculation (indicated by the amplified redistribution of SL-PC to the cytoplasmic leaflet of ejaculated sperm cells compared with epididymal cells). Enhanced reorientation of aminophospholipids to the exoplasmic layer may impair the ability of sperm cells to move within the genital tract and, thus, eventually to fertilize the egg. A suppression of cell motility could result from the interaction of spermatozoa upon exposure of PS in the outer leaflet with the epithelia of the genital tract. Exposure of PS in the outer leaflet in various mammalian cells is known to enhance cellular interactions (for review, see Williamson and Schlegel, 1994). For example, lipid symmetric erythrocytes are more adherent to monolayers of endothelial cells than are erythrocytes of the typical lipid asymmetry, with PS oriented almost exclusively to the cytoplasmic leaflet (Schlegel *et al.*, 1985). Similarly, several studies

have shown that red blood cells, upon incorporation of exogenous PS or exposure of endogenous PS in their outer leaflet, bind more readily to macrophages (Tanaka and Schroit, 1983; McEvoy *et al.*, 1986). The appearance of PS on the surface of apoptotic T lymphocytes also provokes their recognition by macrophages (Verhoven *et al.*, 1995). It is presumed that a similar mechanism mediates phagocytosis of apoptotic spermatogenic cells with PS exposed on their surface by Sertoli cells (Shiratsuchi *et al.*, 1997).

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