Protein-Dependent Translocation of Aminophospholipids and Asymmetric Transbilayer Distribution of Phospholipids in the Plasma Membrane of Ram Sperm Cells†

Karin Müller,‡ Thomas Pomorski,§ Peter Müller,§ Alain Zachowski,‖ and Andreas Herrmann*,§

Institut für Fortpflanzung landwirtschaftlicher Nutztiere Schönow e.V., Bernauer Chaussee 10, D-16321 Schönow, Humboldt-Universität zu Berlin, Mathematisch-Naturwissenschaftliche Fakultät I, Institut für Biologie/Biophysik, Invalidenstrasse 43, D-10115 Berlin, and Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, F-75005 Paris

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ABSTRACT: We have investigated the transbilayer movement of phospholipids in the plasma membrane of ram sperm cells using spin- and fluorescence-labeled lipid analogues. After incorporation into the outer leaflet, phosphatidylcholine (PC) and sphingomyelin (SM) moved slowly to the inner cytoplasmic leaflet, whereas phosphatidylserine (PS) and phosphatidylethanolamine (PE) rapidly disappeared from the exoplasmic monolayer. Variation of the initial velocity of the relocation kinetics vs the amount of analogue incorporated into the membrane suggests a saturability of the transbilayer movement of aminophospholipids. ATP depletion or pretreatment with *N*-ethylmaleimide of ram sperm cells reduced the fast inward motion of PS and PE, indicating a protein-mediated aminophospholipid translocation. The results suggest for the plasma membrane of ram sperm cells the presence of an aminophospholipid translocase and an asymmetric transversal lipid distribution with aminophospholipids preferentially located in the inner leaflet and choline-containing phospholipids in the outer leaflet. The relevance of the transversal segregation of phospholipids for membrane fusion processes occurring during fertilization is discussed.

An asymmetric arrangement of phospholipids between the inner and outer sides is a common property of biological membranes (Op den Kamp, 1979; Zachowski, 1993). The general case is that phosphatidylserine (PS)[1] and phosphatidylethanolamine (PE) are concentrated at the cytoplasmic side, while the choline lipids phosphatidylcholine (PC) and sphingomyelin (SM) are the main components of the outer membrane leaflet. Since the phospholipid asymmetry is stable during the lifespan of the cells, specific mechanisms are required to counterbalance the lipid randomization caused by passive, nonselective transverse diffusion. A specific carrier protein, the aminophospholipid translocase, described originally in human erythrocyte membranes and later on in other plasma membranes of eukaryotic cells, mediates a rapid translocation of aminophospholipids from the outer to the inner leaflet (Seigneuret & Devaux, 1984). The activity of the aminophospholipid translocase is ATP-dependent and sensitive to SH group specific reagents and vanadate (Zachowski et al., 1986; Morrot et al., 1989). Translocation rate of aminophospholipids and steady-state asymmetry of phospholipids depend on the activity of the translocase, which has to be considered as responsible for the maintenance of phospholipid asymmetry in plasma membranes of eukaryotic cells (Herrmann & Müller, 1986; Calvez et al., 1988; Beleznay et al., 1993; Loh & Huestis, 1993).

The existence of such an ATP-consuming process implies that the maintenance of the nonrandom distribution of phospholipids should be important for cell homeostasis, as its abolishment has been shown to have significant effects on membrane properties (Devaux, 1991). Lipid composition significantly affects membrane–membrane aggregation and fusion (Herrmann et al., 1991). Head group associated properties such as charge and hydration extent modulate the close approach of membranes: PC does not favor an interaction because of its bulky hydration layer. PS and PE head groups are less hydrated, allowing a closer contact between membranes. Membrane fusion requires the destabilization of the bilayer structure: PS and PE favor the formation of nonbilayer structures due to their molecular shape and by interacting with Ca^{2+}. Thus, the outer leaflet of eukaryotic plasma membranes is not fusion competent, while the cytoplasmic side, containing PS and PE, is more adapted to fusion with intracellular vesicles. Indeed, it has been recently shown that the external layer of erythrocyte membrane becomes susceptible to fusion with enveloped viruses after destruction of phospholipid asymmetry (Herrmann et al., 1990a, 1991, 1993).

During the reproductive process, mammalian sperm cells have to undergo two crucial events involving membrane fusion processes: (i) fusion of the sperm cell membrane with the outer acrosomal membrane to release acrosomal enzymes and (ii) merging of the postacrosomal parts of the sperm cell

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* Author to whom correspondence should be addressed. FAX: 49-30-28972520. Phone: 49-30-28972830.

‡ Institut für Fortpflanzung landwirtschaftlicher Nutztiere Schönow e. V.

§ Humboldt-Universität zu Berlin.

‖ Institut de Biologie Physico-Chimique.

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[1] Abbreviations: BSA, bovine serum albumin; DFP, diisopropyl fluorophosphate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; ESR, electron spin resonance; DOG, 2-deoxy-D-glucose; mBTS, modified Beltsville thawing solution; NAR, normal acrosomal ridge; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; SM, sphingomyelin; NBD-PC, -PE, and -PS, 1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphatidylcholine, -phosphatidylethanolamine, and -phosphatidylserine; NBD-SM, [*N*-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]sphingosyl]-phosphocholine; SL-PC, -PE, and -PS, 1-palmitoyl-2-(4-doxylpentanoyl)-phosphatidylcholine, -phosphatidylethanolamine, and -phosphatidylserine; SL-SM, *N*-(4-doxylpentanoyl)-*trans*-sphingenyl-1-phosphocholine.

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membrane with the vitelline membrane of the egg (Yanagimachi, 1988; Langlais & Roberts, 1985; Fléchon et al., 1986; Zaneveld et al., 1991). It is tempting to speculate that an asymmetric transverse orientation of lipids and its maintenance by a specific mechanism in sperm cell membranes could play an essential role in the fertilization process. However, the current knowledge on lipid organization of mammalian sperm membranes is very fragmentary (Hinkovska et al., 1986; Hinkovska-Galcheva et al., 1989; Wolf et al., 1990). This is explained by the fragility of sperm cell membranes, which are very sensitive to mechanical and chemical stresses, leading to experimental inaccuracies. Assay of phospholipid orientation in membranes requires methods which allow determination of asymmetry on living cells and do not perturb significantly membrane structure.

For that purpose we have investigated the translocation of phospholipids across the plasma membrane of ram sperm cells using spin- and fluorescence-labeled phospholipids. These analogues mimic quite faithfully the behavior of endogenous phospholipids in biological membranes and incorporate readily from the external solution into the outer leaflet of cell membranes (Morrot et al., 1989; Colleau et al., 1991). Their subsequent transmembrane movement was assessed by two different approaches which allow fast and gentle treatment of membranes. In the case of spin-labeled phospholipids the amount of analogues remaining in the outer leaflet was determined by back exchange on bovine serum albumin (BSA) (Calvez et al., 1988; Morrot et al., 1989). The redistribution of fluorescent NBD-lipids was measured by selective reduction of analogues in the exoplasmic side with dithionite as recently introduced by McIntyre & Sleight (1991) and Pomorski et al. (1994).

MATERIALS AND METHODS

Labeling of Ram Sperm Cells. Ejaculates were obtained from rams (Merino, Texel and Black-head, IFN Schönow e.V.) who give semen continuously over the whole year. All manipulations and experiments were performed at 20 °C. Ejaculates were washed twice (2 min at 2000g followed by 5 min at 3000g) with a modified Beltsville thawing solution (mBTS) containing 205 mM glucose, 20 mM Na-citrate, 15 mM $NaHCO_3$, 10 mM KCl, 1.1 mM $MgCl_2$, and 1 mM EGTA (pH 7). Pellets from different animals were pooled, as the sperm cells of different animals have the same kinetics of redistribution of labeled lipid species (not shown). To prevent hydrolysis of labeled phospholipids, cells were preincubated with diisopropyl fluorophosphate (DFP, Aldrich) at a final concentration of 5 mM. After 5 min of equilibration, 2 vol of cell suspension (2.0×10^9 cells/mL) was mixed with 1 vol of label suspension (label concentration was 1.8 mol % of endogenous membrane phospholipids) representing time zero for all kinetic measurements.

Back-Exchange Assay for Spin-Labeled Phospholipid Analogues. Spin-labeled phospholipids—1-palmitoyl-2-(4-doxylpentanoyl)phosphatidylcholine (SL-PC), -phosphatidylethanolamine (SL-PE), and -phosphatidylserine (SL-PS) and *N*-(4-doxylpentanoyl)-*trans*-sphingenyl-1-phosphocholine (SL-SM)—were prepared as described previously (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 prior to addition to the cell suspension. For back exchange 140-μL samples were transferred at given time points to 60 μL of 3% fatty acid-free BSA (Sigma) on ice and centrifuged after 1 min of incubation (30 s at 12000*g*). The amount of probe present in the supernatant was estimated from its ESR spectrum intensity after reoxidation by ferricyanide (10 mM) using a Bruker ECS 106 spectrometer.

Internal Reduction of Spin-Labeled Analogues. To assess differently the inward translocation of spin-labeled lipids, we have continuously monitored the intensity of the ESR spectrum at room temperature, as the probes are reduced on the inner layer owing to the reducing properties of the sperm cytoplasm, leading to a decrease of the signal. The rate constant of internal reduction was measured in separate experiments by measuring the signal loss after sonification of pelleted cells.

Dithionite Assay with Fluorescence-Labeled Phospholipid Analogues. Phospholipid analogues with an NBD group on the short β-fatty acid chain were synthesized as described by Colleau et al. (1991). For labeling, cells were treated as described for spin-labeled phospholipids. Since fluorescence of NBD fluorophores in the micellar structures is quenched, the incorporation of analogues into the plasma membrane can be followed by the relief of fluorescence self-quenching. In the case of NBD-PE, the nonincorporated amount was removed after 2 min by washing (20 s at 12000*g*). The redistribution of NBD labels between the two leaflets of the sperm cell membrane was determined by the selective reduction of NBD-lipids in the outer one using dithionite as described recently (Pomorski et al., 1994). At given time points 50-μL samples were transferred to 2 mL of mBTS. The decrease of fluorescence intensity at 540 nm (excitation at 470 nm) was monitored (Shimadzu RF 5001 PC spectrometer) in the presence of 22 mM dithionite added from a stock solution. The difference between initial and remaining fluorescence intensity is a measure for the amount of label located in the outer leaflet of the cell membrane. Treatment with Triton X-100 (final concentration, 0.5%) renders all label accessible to dithionite, abolishing the fluorescence totally.

Assessment of Sperm Cell Quality. The normal acrosomal ridge (NAR) of an intact acrosome can be detected under phase-contrast optics at a magnification of 1000×. Simultaneously, differentiation of cell status was performed by fluorescence microscopy. To this end, sperm cells were stained with the fluorescent dye Hoechst 33258, which rapidly penetrates defective cells. Briefly, 5-μL samples were diluted 5 times with mBTS and then fixed with 2% formol in mBTS (1:1, vol:vol). After dilution with mBTS, cells were stained with Hoechst 33258 according to de Leeuw et al. (1991) and wet slides were prepared. For the establishment of motility, sperm cells were stepwise diluted 1:30 with mBTS and transferred into a prewarmed (37 °C) Makler chamber. Motility parameters were revealed by computer-assisted motion analysis (Strömberg-Mika) from four visual fields in two chambers per sample (200–300 cells) according to Seibert (1988). The determination of cellular ATP content was performed on a BioOrbit-Luminometer using an ATP monitoring kit (Colora, Germany). As can be seen in Table 1, all ejaculates were of high initial quality. Neither spin- nor fluorescence-labeled analogues had an effect on sperm cell motility and integrity. Moreover, there is no deterioration of sperm cell quality parameters within the time course of the experiments. Neither NEM treatment nor ATP depletion affected the integrity of membrane structure as proved by the percentage of cells with a NAR and not stained by Hoechst 33258 (data not shown).

Miscellaneous. Data for the fast redistribution of aminophospholipid analogues were exponentially fitted (SigmaPlot; see Results). Results are expressed as mean ± standard deviation, with n being the number of experiments.

Table 1: Sperm Quality during Incubations with Spin-Labeled Phospholipid Analogues[a]

	NAR (%)					motile cells (%)			VAP (μm/s)		
	0 min	60 min	(*n*)	H-neg (%), 60 min	(*n*)	30 min	90 min	(*n*)	30 min	90 min	(*n*)
SL-PS	84 ± 7	84 ± 4	(5)	84 ± 9	(3)	40 ± 16	58 ± 14	(5)	109 ± 11	120 ± 17	(5)
SL-PC	86 ± 4	86 ± 6	(5)	86 ± 6	(3)	46 ± 13	51 ± 19	(5)	116 ± 17	106 ± 31	(5)
SL-PE	79 ± 6	83 ± 8	(3)								
SL-SM	79 ± 2	81 ± 6	(3)								
control	84 ± 6	85 ± 6	(5)	90 ± 9	(3)	48 ± 10	45 ± 12	(5)	112 ± 5	103 ± 17	(5)

[a] NAR, percentage of cells with a normal acrosomal ridge; H-neg, percentage of cells not stained by Hoechst 33258; VAP, average path velocity of all motile cells in the sample. Data are given as average ± standard deviation; *n* is the number of experiments.

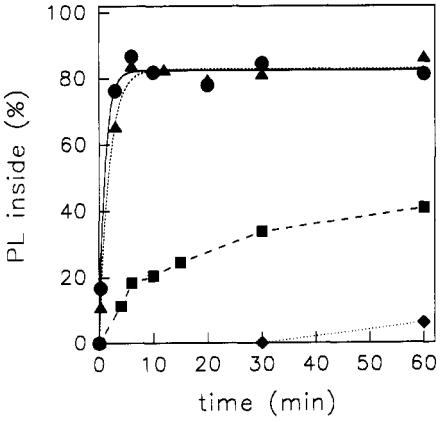

FIGURE 1: Redistribution of spin-labeled phospholipid analogues in ram sperm cells at 20 °C. The fraction of lipids in the inner leaflet was determined by the back-exchange assay as described in Materials and Methods; ●, SL-PS; ▲, SL-PE; ■, SL-PC; ◆, SL-SM.

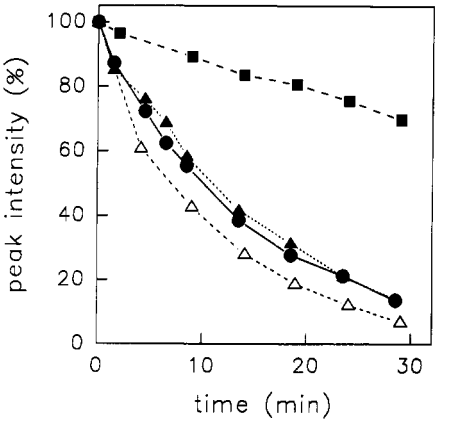

FIGURE 2: Reduction of the ESR signals from spin-labeled phospholipid analogues incorporated in ram sperm cells at 20 °C. For symbols used, see Figure 1. The loss of signal intensity after sonication of sperm cells is only shown for SL-PE (△) for the sake of clarity.

RESULTS

Transmembrane Movement of Spin-Labeled Phospholipid Analogues. Total uptake of the spin-labeled analogues into membranes occurs within 15 s after probe addition. This was the shortest time elapsed between mixing and recording of ESR spectra. Spectra of membrane-associated label were clearly different from those of probes dissolved in aqueous buffer (Seigneuret et al., 1984). Back-exchange assay performed immediately after membrane labeling showed that ≥95% of zwitterionic analogues SL-PC and SL-SM can be extracted from the outer leaflet. The fast inward translocation of aminophospholipid analogues (see below) did not allow this measurement. However, if the rate of translocation was reduced (ATP depletion or NEM treatment), ≥95% of the SL-PS and SL-PE was extractable by BSA from the outer leaflet immediately after membrane labeling. Owing to these findings, we fitted all translocation kinetics of the aminophospholipid analogues to time zero in order to get the amount of analogues initially incorporated into the outer leaflet. This estimated quantity was in agreement with the known amount of analogue added to the cell suspension.

Figure 1 shows characteristic kinetics of the redistribution of spin-labeled analogues from the outer to the inner half of ram sperm plasma membranes at room temperature. The transbilayer movement differs clearly between the four labels. The internalization of the aminophospholipid SL-PS is fast with a half-time on the order of 1 min. After 5 min nearly 80% of PS analogues were internalized and the steady-state distribution was on the order of 80.2 ± 4.1% (n = 5) in the inner leaflet. Similar kinetics and plateau (83.8 ± 2.6%, n = 3) were found for SL-PE. The stable steady-state distribution of both analogues suggests that label exchange between the cytoplasmic half of the plasma membrane and intracellular membranes such as the underlying acrosomal membrane is negligible. Moreover, we have no indication of endocytic uptake of analogues mimicking a redistribution of label to the inner half.

In contrast to the aminophospholipids, SL-PC and SL-SM moved only slowly to the cytoplasmic half. After 1 h, 59.7 ± 8.7% (n = 5) of PC analogues and 93.9 or 88.2% (n = 2) of SM analogues could be removed by BSA from the outer membrane leaflet.

To obtain additional evidence for the different redistribution behavior of spin-labeled aminophospholipids to the inner leaflet, the reduction of the NO group by intracellular redox systems was measured. For this purpose, the intensity of the ESR signal of probes incorporated into the sperm membrane was continuously recorded. SL-PS- and SL-PE-associated ESR signals decreased rapidly, while the intensity of the SL-PC signal decreased more slowly (Figure 2), which is in accordance with the back-exchange assay. To preclude that the difference in reduction kinetics between analogues arose from a specific sensitivity of PS or PE analogue to redox systems, we recorded the reduction kinetics after disruption of sperm cells by ultrasonication. In that case we observed a continuous decline of ESR intensity whatever the spin-labeled analogue.

To further assess that SL-PS and SL-PE move to the inner leaflet with approximately the same velocity, the outside–inside translocation of both analogues in the concentration range from 0.2 to 4.5 mol % of endogenous plasma membrane phospholipids was investigated. Initial velocities of inward motion were obtained by exponential fitting of the redistribution kinetics. Higher label concentrations were not used in order to prevent perturbations of membrane stability. Within the experimental error the concentration dependence of the initial velocity of inward motion was the same for both spin-labeled aminophospholipids (Figure 3). Although due to experimental limitations not rigorously shown, the data suggested a saturable behavior (Figure 3). The estimated

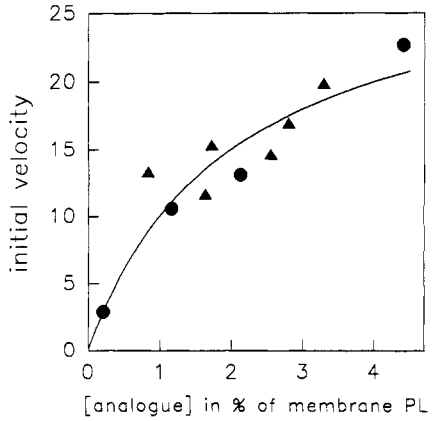

FIGURE 3: Initial velocity of SL-PS (●) and SL-PE (▲) reorientation in ram sperm cell membranes as a function of analogue concentration. Cell suspensions contained about 1.0×10^9 cells/mL. The rate was expressed in nanomoles of analogue/minute/(milligram of membrane protein). The curve was obtained by fitting all data to the Michaelis–Menten equation.

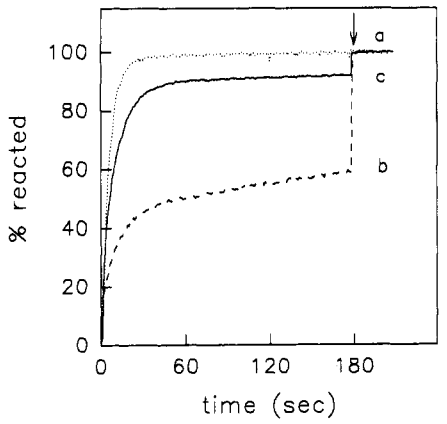

FIGURE 4: Reduction of fluorescence intensity of NBD-labeled phospholipid analogues in ram sperm cells by dithionite at 20 °C: (a) NBD-PC immediately after labeling; (b) NBD-PS 60 min after labeling; (c) NBD-PC 60 min after labeling. Labeling was carried out as described in Materials and Methods. At $t = 0$, dithionite was added to a final concentration of 22 mM and the fluorescence was recorded. In order to destroy membrane integrity, Triton X-100 was added to a final concentration of 0.5% after 3 min (arrow).

maximum reorientation rate was 29.8 nmol of analogue/min/(mg of membrane protein), and the apparent Michaelis–Menten constant (K_m) was 2.0% of the membrane lipids.

Transmembrane Movement of Fluorescence-Labeled Phospholipid Analogues. Except for NBD-PE (see Material and Methods), ≥95% of the labels were incorporated within 2 min into the cell membrane as revealed by dequenching measurements (see Materials and Methods). Immediately after labeling of membranes, most of the fluorescence is rapidly destroyed upon addition of dithionite independent of the head group (Figure 4, curve a, NBD-PC). However, after longer incubation at room temperature only part of the NBD-PS and NBD-PE was accessible to dithionite; after a fast fluorescence decrease the remaining fluorescence declined only slowly, indicating a slow uptake of dithionite into the sperm cells (Figure 4, curve b, NBD-PS, 60 min after labeling). This remaining fluorescence corresponds to NBD analogues at the inner leaflet of the sperm cell membrane since it disappeared rapidly after disruption of the membrane by Triton X-100. In contrast to fluorescent aminophospholipids, NBD-PC- and NBD-SM-associated fluorescence disappeared rapidly after dithionite treatment at 60 min after labeling, suggesting that analogues are still in the exoplasmic layer (Figure 4; curve c, NBD-PC, 60 min after labeling).

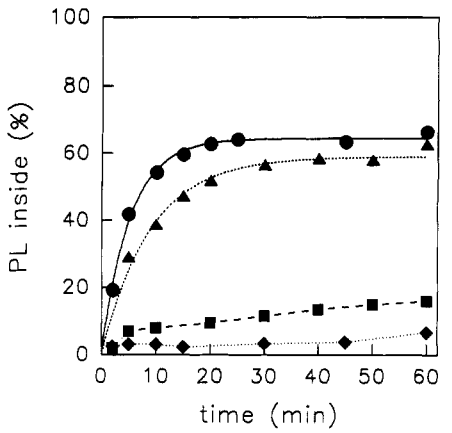

FIGURE 5: Transmembrane redistribution of NBD-labeled phospholipid analogues in ram sperm cells at 20 °C. The fraction of lipids in the inner leaflet was determined by the dithionite assay as described in Materials and Methods. For symbols used, see Figure 1. Note that the first point could only be measured 2 min (NBD-PS, NBD-PC, NBD-SM) or 5 min (NBD-PE) after labeling due to the experimental setup.

Figure 5 shows the amount of NBD analogues not accessible to dithionite as a function of time. Again, the redistribution of aminophospholipid analogues was rapid, contrary to that of zwitterionic probes NBD-PC and NBD-SM. The half-times of redistribution for NBD-PS and NBD-PE are about 3 and 6 min, respectively. The plateaus of NBD-PS corresponding to 63.4 and 62.5% ($n = 2$) and of NBD-PE corresponding to 58.9 and 60.2% ($n = 2$) of probe in the cytoplasmic leaflet suggest a steady-state distribution between both halves of the plasma membrane without significant exchange of label with intracellular compartments. Due to the very slow redistribution of NBD-PC and NBD-SM it was impossible to estimate the final distribution of these analogues across the plasma membrane. After 1 h, 86.2 and 88.1% ($n = 2$) of NBD-PC and 93.4 and 94.3% ($n = 2$) of NBD-SM was still accessible to dithionite.

Influence of NEM Treatment and ATP Depletion on Redistribution of Analogues. NEM treatment of sperm cells (2 mM NEM for 30 min) resulted in a superposition of the translocation curve of PS analogues on that of PC analogues for spin- as well as fluorescence-labeled lipids (Figure 6). The kinetics of SL- or NBD-PC transmembrane motion were unchanged after incubation with NEM.

Cell ATP content was reduced by a preincubation of sperm cells (20 °C) in mBTS medium under conditions inhibiting both glycolysis (replacement of glucose by 2-deoxy-D-glucose, DOG) and respiration (5 mM NaN_3). After 90 min the ATP content dropped to about 5% (0.6 ± 0.5 nmol/10^8 cells, $n = 3$) of that in control cells in mBTS with glucose (11.2 ± 3.1 nmol/10^8 cells, $n = 3$). The initial velocities of SL-PS and SL-PE inward translocation were reduced to 23 or 34% and 24 or 28% ($n = 2$), respectively, of control values (Figure 7). However, the plateaus derived from exponential curve fits are only slightly lowered by less than 5%. When only glycolysis was inhibited, ATP reduction (26% of the amount in the control cells, 2.9 ± 0.5 nmol/10^8 cells, $n = 3$) was not sufficient to affect inward movement of aminophospholipid analogues. No influence of ATP diminution on SL-PC redistribution could be detected even if respiration was hindered.

It is important to note that NEM treatment caused a comparable depletion of intracellular ATP to about 2.5% of the amount in control cells. However, ATP depletion alone cannot explain the effect of NEM pretreatment because PS translocation is much more affected than in ATP-depleted

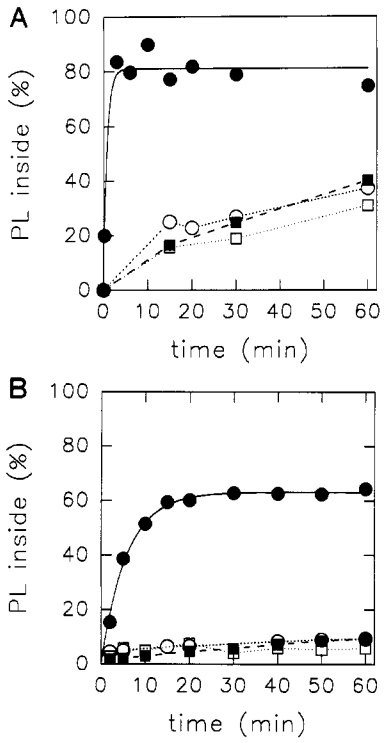

FIGURE 6: Influence of NEM treatment on the redistribution of phospholipid analogues in ram sperm cells. Cells were preincubated for 30 min in the presence of 2 mM NEM. The fraction of spin- (A) and fluorescence-labeled lipids (B) in the inner leaflet was determined using the back-exchange assay (A) and the dithionite assay (B), respectively: ●, SL-PS and NBD-PS control; ○, SL-PS and NBD-PS, NEM treated; ■, SL-PC and NBD-PC control; □, SL-PC and NBD-PC, NEM treated.

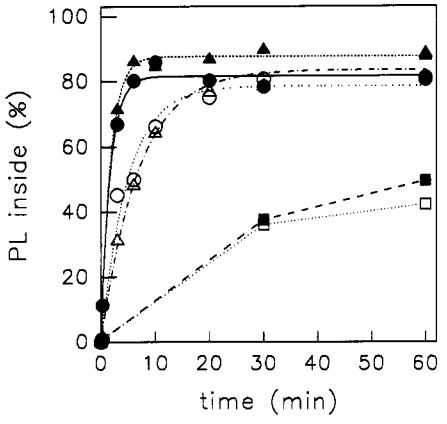

FIGURE 7: Influence of ATP depletion on the redistribution of spin-labeled phospholipids. ATP depletion and estimation of the fraction of lipid analogues in the inner leaflet using the back-exchange assay were performed as described in Materials and Methods; filled symbols, control cells; open symbols, ATP depleted cells; ●, ○, SL-PS; ▲, △, SL-PE; ■, □, SL-PC.

cells (see above). This argues for a specific effect of NEM. A similar observation on the influence of NEM on ATP content has been described very recently for endothelial cells (Julien et al., 1993).

DISCUSSION

The transmembrane movement of spin- as well as fluorescence-labeled phospholipid analogues in ram sperm cell membrane resembles that found in eukaryotic blood cells [see Zachowski (1993)], platelets (Sune et al., 1987), fibroblasts (Martin & Pagano, 1987; El-Hage Chahine et al., 1993), chromaffin granules (Zachowski et al., 1989), and synaptosomes (Zachowski & Morot-Gaudry Talarmain, 1990): whereas PC and SM disappeared slowly from the exoplasmic leaflet, PS and PE moved rapidly to the cytoplasmic half. The translocation of the aminophospholipids is mediated by an ATP-dependent protein, as (i) ATP depletion suppressed the fast aminophospholipid redistribution and (ii) pretreatment with an SH-group-specific reagent (NEM) drastically reduced the inward movement of PS analogues to the level of the slow passage of PC. Reasonably, the aminophospholipid translocase (Seigneuret & Devaux, 1984) has to be considered as the carrier system in mammalian sperm cells. PC and SM translocation was not significantly affected by either NEM addition or ATP depletion. This suggests that these zwitterionic phospholipids cross the plasma membrane by passive diffusion as observed for other plasma membranes. Recently, we found similar results for translocation and distribution of spin-labeled phospholipid analogues in plasma membranes of fish sperm cells (Müller et al., 1994).

A fast translocation of aminophospholipid analogues to the cytoplasmic half and a slow passive flip-flop of zwitterionic phospholipids were evidenced with SL analogues. First, while SL-PS and SL-PE became very rapidly inaccessible to BSA, about 60% of SL-PC and 90% of SL-SM could be extracted from the outer layer 1 h after labeling. It was impossible to resolve appropriately the initial rate of aminophospholipid analogue movement at room temperature. However, redistribution of probes was not performed at a lower temperature owing to the cell integrity with respect to cooling (Watson, 1981; Hammerstedt et al., 1990). Second, reduction of the nitroxide group on the cytoplasmic half by intracellular redox systems was fast for SL-PS and SL-PE and slow for SL-PC. However, compared with the results of the back-exchange assay, the characteristic time of inward translocation appeared to be longer. This can be explained by the rate constant of internal reduction, which was about 7 min and acted as the rate-limiting step of this assay.

Results of spin-labeling experiments were confirmed by the redistribution of fluorescent analogues. The dithionite assay could be applied to sperm cells and allowed a quantitative description of redistribution kinetics. As the plasma membrane of ram sperm cells was almost impermeable to dithionite at room temperature, only NBD analogues on the outer leaflet were modified by dithionite. Data obtained with fluorescent probes were in qualitative agreement with those obtained with spin-labeled analogues, but significant differences between both initial rates of translocation and transmembrane equilibrium of spin- and fluorescence-labeled aminophospholipids were established. The observed differences may be the reflection of different abilities—according to the chemical structure of the probes, e.g., the label moiety—to substitute for natural phospholipids. Indeed, similar differences between SL and NBD analogues have been observed for human erythrocyte (Colleau et al., 1991; Pomorski et al., 1994) and fibroblast membrane (T. Pomorski et al., unpublished results). For instance, translocation of NBD-PS was significantly slower than that of SL-PS. This has been ascribed to a reduced affinity of the aminophospholipid translocase for the short-chain fluorescent lipids (Colleau et al., 1991).

The aminophospholipid translocase has a higher affinity for PS than for PE; e.g., in the human erythrocyte membrane the ratio of the K_m values of PE and PS is about 10 (Zachowski et al., 1986). Such a difference between affinities for PS and PE could not be established in ram sperm cell membranes: (i) No difference in the initial velocity of transbilayer movement of SL-PS and SL-PE could be found (Figures 1–3).

(ii) ATP depletion had a similar effect on the inward motion of both analogues (Figure 7). (iii) In ram sperm cell membranes the C_6-NBD-PE analogue was translocated to the inner leaflet nearly as rapidly as the C_6-NBD-PS was (Figure 5). This result is opposed to the situation encountered in human red blood cells where NBD-PE was not translocated by the translocase at all (Colleau et al., 1991).

Inward translocation of aminophospholipid analogues in ram sperm cell membranes is significantly faster than in human erythrocytes (Zachowski et al., 1986) but occurs at a rate close to that reported in K 562 cells (Cribier et al., 1993). Several reasons may account for this difference. (i) A higher activity of the translocase could be associated with a higher fluidity of cell membranes. However, comparison of the line shapes of EPR spectra of SL-PC immediately after insertion into erythrocyte and sperm cell membranes did not show any different rotational mobilities (spectra not shown). (ii) A greater number of copies of the protein could be mediating the ATP-dependent translocation. This may account also for the rather gradual influence of ATP depletion on the translocation activity (see above). However, to answer this question would require more information on the nature and organization of the protein in membranes. Taking into account the structure of sperm cells (e.g., head, tail), one may ask whether the lateral organization of the aminophospholipid transporter is homogenous in the sperm plasma membrane.

Do the differential movements of labeled lipid analogues reflect an asymmetric distribution of phospholipids in sperm plasma membranes? Differential disappearance of labeled analogues gives no direct measure of an asymmetric steady-state distribution between the outer and inner monolayers as an equilibrium of probe between the cytoplasmic half of the plasma membrane and intracellular membranes might cause a continuous removal of labeled lipids from the plasma membrane. However, present data give clues for the transverse distribution of phospholipids in those membranes. Since such an ATP-dependent translocation of aminophospholipids has been shown to maintain lipid asymmetry in human erythrocyte membranes, we may anticipate that a similar transverse distribution exists in sperm cell membranes with aminophospholipids preferentially located on the inner half and choline phospholipids in the outer leaflet. This hypothesis is sustained by the plateau observed for distribution of both fluorescence- and spin-labeled aminophospholipids, suggesting that a removal of label from the plasma membrane in the time course of the experiment was negligible.

It has been shown that the membrane skeleton of human red blood cells plays only a minor role, if any at all, in maintaining phospholipid asymmetry (Herrmann & Müller, 1986; Calvez et al., 1988; Beleznay et al., 1993; Loh & Huestis, 1993). However, at the present stage our data do not allow us to exclude a possible role of the cytoskeleton of sperm cells in transverse lipid arrangement (Jamil, 1984; Kann et al., 1993). Further studies are warranted on this subject.

One may wonder why the final plateau of transversal distribution of SL-PS and SL-PE was not affected by the dramatic reduction of the ATP level to 5–10% of the control value. Even if the fast inward translocation of spin-labeled aminophospholipids was slowed upon depletion of intracellular ATP, it was still significantly faster than that of SL-PC or SL-SM. Moreover, in human erythrocytes, a similar ATP depletion affected the redistribution of aminophospholipids more strikingly (Calvez et al., 1988), although the intracellular ATP concentrations of fresh washed red blood cells and sperm cells are comparable (about 12 nmol/10^8 red blood cells and 14.1 ± 4.2 nmol/10^8 ram sperm cells, $n = 7$). This suggests that the remaining amount of ATP in ram sperm cells is still sufficient to maintain a high inward translocation of aminophospholipids. Unfortunately, it was not possible to decrease further the ATP level without affecting ram sperm cell integrity. It is important to note that a partial loss of the translocase activity does not necessarily cause a more symmetric transversal phospholipid distribution, as an inhibition of about 80% of the translocase activity in red blood cell membranes did not affect the plateau of the inside–outside distribution of SL-PS (Herrmann et al., 1990b). This can be rationalized by kinetic models describing the asymmetric distribution of phospholipids in eukaryotic plasma membranes on the basis of the translocase activity (Herrmann & Müller, 1986; Brumen et al., 1993).

Such an asymmetric phospholipid distribution supports the hypothesis that the cytoplasmic leaflet of eukaryotic plasma membranes is fusion-competent while the outer surface is not. As mammalian sperm cells undergo fusion between the plasma and acrosomal membranes during the acrosome reaction, the preferential localization of the fusion-supporting aminophospholipids might play a significant role in the acrosome reaction. Even if specific proteins are involved in the fusion process (Langlais & Roberts, 1985; Wassarman, 1992), one may surmise that the lipid composition of the outer acrosomal membrane is optimal to favor fusion. However, further investigations on the transverse distribution of phospholipids in the acrosomal membrane and its regulation during capacitation/acrosome reaction should be entertained to outline the role of these phospholipids in fertilization.

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