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Changes in the lipid content of boar sperm plasma membranes during epididymal maturation

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Plasma membranes of boar sperm from caput, corpus and cauda of the epididymis were purified by differential- and sucrose-density equilibrium centrifugation and were found to yield a single band at a density of 1.13 g/cm^3. This fraction was enriched in acid and alkaline phosphatase, 5′-nucleotidase and ($Na^+ + K^+$)-ATPase activities, whereas it contained minimal amounts of hyaluronidase and *N*-acetylglucosaminidase and no succinic acid dehydrogenase activities. The plasma membrane of caput, corpus and cauda sperm had the same phospholipid/protein and cholesterol/phospholipid ratios but yielded different amounts of protein and individual lipid classes. Several changes in the plasma membrane were observed during transit of sperm through the epididymis. Within the phospholipid class a decrease in the percentage of phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol was detected accompanied by an increase in amount of phosphatidylcholine, sphingomyelin and polyphosphoinositides. In the other lipid classes there was a decrease in the amount of free fatty acid and the major glycolipid. The amount of cholesterol decreased, while the amount of desmosterol and cholesterol sulfate increased. There was an increase in the amount of diacylglycerol. In addition, the changes in the fatty acid composition of the total membrane lipid and each phospholipid were determined. The above changes in the lipid composition of the plasma membrane during epididymal maturation may help to explain the decreased resistance to cold shock and changes in membrane fluidity of sperm during transit in the epididymis.

Introduction

Mammalian spermatozoa do not have the ability to fertilize the egg upon leaving the testis, but they develop fertilizing capacity as they pass through the epididymis. This process, called the epididymal maturation of spermatozoa, includes various morphological, physiological and biochemical changes in the sperm (for review, see Ref. 1), such as the acquisition of sperm motility and alterations in the properties of the plasma membrane [2]. The changes in the sperm plasma membrane reported to occur during epididymal maturation include changes in the distribution of proteins and glycoproteins, alteration of the surface charge and changes in certain enzymatic activities [1].

Reports on changes of the sperm lipid composition during epididymal maturation have been based on lipid extracts of whole sperm. A decrease in the amount of sperm lipid during transit through the

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TMS, trimethylsilyl; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PIP, phosphatidylinositol-4′-phosphate; PIP_2, phosphatidylinositol-4′,5′-bisphosphate.

epididymis has been reported in boar [3,4], bull [5], ram [6,7] and rat [7,8], with the exception of choline plasmalogens which remain the same or increase [8,9]. This decrease in the lipid content has been ascribed to the utilization of lipids as energy sources. Furthermore, loss of lipid during maturation of sperm may explain the greater sensitivity of ejaculated spermatozoa to cold shock than either testicular or epididymal spermatozoa [10–12] and decreased membrane fluidity shown by fluorescence polarization spectroscopy [13]. Both phenomena are presumed to involve the plasma membrane, but the lipid analyses have been done only on whole sperm and not on isolated plasma membrane.

To evaluate the relation between changes in sperm lipids with changes in the properties of the plasma membrane during maturation, it is necessary to first isolate sperm plasma membrane and then quantitate the membrane lipids in sperm from different portions of the epididymis. In the present paper we report on the purification of plasma membrane of boar sperm from the caput, corpus and cauda of the epididymis, the biochemical characterization of the purified membranes and the quantitation of plasma membrane lipid of sperm from the three parts of the epididymis.

Materials and Methods

Materials

Lipid standards and GLC columns were purchased from Supelco Inc. Radioactively labelled lipid standards were obtained from Amersham. Organic solvents were of ACS or better grade and were from either Fischer Scientific or J.T. Baker Chemical Co. Other chemicals were from Calbiochem-Boehring or Sigma Chemical Co.

Sperm plasma membrane preparation

Epididymides were obtained from freshly slaughtered boars (American Meat Packing Corp., Chicago) and laboratory processing was initiated within 1 h. The epididymides were divided into sections B to F_b as described by Zimmerman et al. [14]; sections B to C were cut as caput, sections D_b to E_a as corpus and sections F_a to F_b as cauda. Sections D_a and E_b were not used thereby preventing mixing of sperm of different stages of maturation. The epididymides segments were minced with a razor blade in 10 mM Hepes/0.25 M sucrose/1 mM EDTA (pH 7.5). Sperm were decanted from the tissue and strained through a nylon mesh. An aliquot of the sperm preparation was centrifuged through a 30–80% linear gradient of Percoll at $4000 \times g$ for 20 min, to assess the uniformity of the sperm suspension. After centrifugation the gradients were fractionated and the sperm was localized by measuring the turbidity at 600 nm. The remaining sperm suspension was further processed as described by Soucek and Vary [15] except that the washed sperm suspension was homogenized by treatment with nitrogen cavitation in a Parr bomb at 650 lb/in^2 for 10 min [16]. In addition, all the buffers used for the plasma membrane preparation contained 1 mM EDTA.

Lipid analysis

Lipids were extracted as described by Cohen et al. [17]. The recovery was estimated to be $90 \pm 5\%$ based on the recovery of either [^{3}H]phosphatidylinositol or, in the case of sterol, [^{3}H]cholesterol, added at the beginning of the extraction. Lipid extracts were stored at $-20°C$ in chloroform/methanol (2:1) containing 0.01% butylated hydroxytoluene to eliminate lipid oxidation. For quantitation of the total phospholipid, the phospholipid phosphate was liberated by either ashing an aliquot of the lipid extract [18] or digesting phospholipid scraped from TLC plates in 70% perchloric acid [19], and the phosphate was assayed by the method of Chen et al. [18].

Lipids were fractionated on 0.3-mm-thick silica gel H TLC plates activated by heating at 110°C for 30 min and developed according to Freeman and West [20]. The lipids were located by spraying with 0.2% 2′,7′-dichlorofluorescein in ethanol and illumination with an ultraviolet light. They were identified by their chromatographic mobilities relative to lipid standards and by specific sprays (75% H_2SO_4 for sterols, ninhydrin for aminolipids, molybdenum blue for phospholipids and orcinol for glycolipids [21]). The areas on the TLC plate corresponding to individual lipids (except cholesterol) were scraped into screw-cap test-tubes containing tricosanoic acid as an internal standard. The lipids were transesterified, the resulting methyl esters extracted and the fatty acid composi-

tion determined by GLC on a Varian model 3700 chromatograph equipped with a 2 m × 2 mm i.d. stainless steel column packed with SP-2330 on 100/120 mesh Chromosorb, as previously described [22]. The column was maintained at 190°C while the injector and detector temperatures were at 250°C and 300°C, respectively, and the carrier gas was nitrogen at a flow rate of 30 ml/min. The fatty acid methyl esters were identified by comparison of their equivalent chain length with standards and by mass spectrometry on a Finnigan MAT 112 S spectrometer equipped with a 30 m × 0.25 mm i.d. fused silica capillary column coated with SP-2330. The spectrometer was operated with electron multiplier at 1.5 kV, emission current +1.5 mA and an electron energy of 70 eV. They were quantitated by comparing the peak area relative to the internal standard, methyl tricosanoate, with the aid of an integrator (Hewlett Packard Model, 33904).

Phospholipids were fractionated by two-dimensional TLC on layers of silica gel H (0.3 mm thick) impreganted with 2.5% potassium oxalate. The plates were developed in chloroform/methanol/ammonium hydroxide (60 : 30 : 5) in the first dimension and chloroform/ acetone/ methanol/ acetic acid/H_2O (40 : 15 : 13 : 12 : 8) in the second dimension [23]. Phospholipids were identified by comparing their chromatographic mobilities with those of standards. For quantitation, spots corresponding to each phospholipid were scraped into test-tubes, digested and analyzed for the liberated inorganic phosphate as described above. Fatty acid compositions of the phospholipids were determined as described previously.

For the estimation of the alkylacyl and alk-1-enylacyl forms of the phosphoglycerides, phospholipids purified by two-dimensional TLC were hydrolysed with *Bacillus cereus* phospholipase C [24]. The disubstituted glycerols formed were extracted and fractionated by TLC as described [9]. This produced two fractions, one of which contained both alk-1-enylacyl and alkylacyl glycerols and the second contained the diacylglycerols. The disubstituted glycerols were transesterified and analyzed by GLC as described above. The above method resulted in estimation of the diacyl and the combined alkylacyl and alk-1-enylacyl fractions of the phospholipids. The amount of the alk-1-enylacyl form of each phospholipid was estimated as described by Owens [25] except that chloroform/methanol/ammonia (60 : 30 : 5) and chloroform/acetone/methanol/acetic acid/H_2O (40 : 15 : 13 : 12 : 8) were used for the development of the plate in the first and second dimension, respectively.

For sterol analysis, an aliquot of the lipid extract was applied on activated silica H TLC plates and the plates were developed in chloroform/methanol/acetic acid (80 : 20 : 2). The sterols were located by spraying with 75% H_2SO_4 and heating at 110°C for 5 min or with iodine. The areas on a plate that corresponded to sterol were scraped into test-tubes, 50 nmol of 5-α-cholestane were added as an internal standard and the sterols were extracted with chloroform and analyzed by GLC. The chromatograph was equipped with a 1 m × 2 mm i.d. stainless steel column packed with SP-2250 on 100/120 Supelcoport. The carrier gas was N_2 at a flow rate of 30 ml/min. The column was maintained at 250°C, while the injection port and the detector were at 300°C. Individual sterols were identified by comparing the retention time with those of known sterols, and mass spectrometry. The amount of sterol in each sample was determined by comparing the integrated peak areas to the peak area of the standard 5-α-cholestane. Mass spectrometry was performed on a Finnigan 4510 spectrometer equipped with a 15 m × 0.25 mm i.d. fused silica capillary column coated with methyl silicone. The carrier gas was helium at a flow rate of 0.7 ml/min. The mass spectrometer was operated with electron multiplier at 1 kV, emission current at −0.25 mA and an electron energy of 70 eV.

For the quantitation of the sulfated sterols, the areas on the plates corresponding to cholesterol sulfate were scraped into screw-cap test-tubes containing 5-α-cholestane, solvolyzed [26] and the liberated sterols were analyzed by GLC as described above for the free sterols.

For the determination of the glycolipid structure, the total lipid extract was applied on a silicic acid column (1 g, activated at 110°C for 2 h). The column was sequentially eluted with chloroform (neutral lipid fraction), acetone (glycolipid fraction) and methanol (phospholipid fraction) [9]. The acetone fraction was chromatographed in two

TLC systems which were silica gel H plates impregnated with 2.5% of potassium oxalate and developed in chloroform/acetone/methanol/acetic acid/H_2O (40:15:13:12:8) and silica gel H plates developed in chloroform/methanol/H_2O (80:20:2). Lipid-bound sulfate was estimated by the method of Kean [27]. Commercial bovine brain sulfatides were used for the standard curve.

The fatty acid and sugar composition of the glycolipid fraction was determined after solvolysis with 0.75 M HCl in methanol at 80°C for 24 h. The fatty acid methyl esters formed were extracted with hexane and analyzed by GLC as described above. The TMS derivatives of the sugars and glyceryl ethers were made from the remaining methanolic phase after extraction with hexane, as described by Ishizuka et al. [28], except that 50 μl of bis(trimethylsilyl)trifluoroacetamide and 50 μl of pyridine were added to each sample and the tube was heated at 130°C for 3 h. The TMS derivatives were analyzed by GLC on a 3 m × 2 mm i.d., stainless steel column packed with SE-30 on 80/100 Supelcoport. Identification was based on comparison of the retention times with those of standard sugars and glyceryl ethers treated the same way as the sample. The standards used for the quantitation of the TMS derivatives of the glycolipid sugar and the glyceryl ether were TMS derivatives of glucose and 1-*O*-octadecyl-DL-glycerol, respectively. After the structural determination, the routine estimation of the amount of the glycolipid in each sample was based upon isolation of the glycolipid by the two-dimensional TLC system described above and estimation of the esterified fatty acid by GLC or of the bound sulfate by the method of Kean [27]. Both methods routinely gave the same results within a ±5% error.

Biochemical assays

Acid (EC 3.1.3.2) and alkaline (EC 3.1.3.1) phosphatases were determined as described by Soucek and Vary [15]. Succinic acid dehydrogenase (EC 1.3.99.1) was assayed as previously described [29] after incubating the membranes at 37°C in 0.1% Triton X-100 for 1 h to release mitochondrial enzymes.

5′-Nucleotidase (EC 3.1.3.5) was assayed by the method of Gerlach and Hiby [30]. The amount of phosphate liberated was determined by the malachite green method of Lanzetta et al. [19]. For each component of the enzyme reaction mixture the optimum concentration was determined; nickel was not used as an inhibitor in the assays [30] because it did not inhibit 5′-nucleotidase of boar sperm (Mitchell and Vary, unpublished observations).

$(Na^+ + K^+)$-ATPase (EC 6.3.1.3) was assayed by a modification of the method of Past and Sen [31] except that $MgCl_2$ and silicotungstic acid were not used. The activity was calculated as the amount of ouabain-sensitive phosphatase activity.

Hyaluronidase (EC 3.2.1.35) and *N*-acetylglucosaminidase (EC 3.2.1.30) were estimated by the methods of Zaneveld et al. [32], and Khar and Anand [33], respectively. Protein concentration was measured, after precipitation with cold 5% trichloroacetic acid to remove amines, by the method of Lowry et al. [34] using bovine serum albumin as the standard. DNA and RNA were measured by the diphenylamine [35] and orcinol [36] colorimetric assays, respectively.

Results

Sperm were isolated from three regions of the boar epididymis by standard procedures [16,37,38] and an aliquot of the sperm preparations was centrifuged on linear Percoll gradients to assess the cell uniformity. Turbidity measurements of the fractionated gradients suggested that the sperm of caput, corpus and cauda epididymis had a density of 1.09 g/cm^3 and there was no significant (less than 3%) contamination of the sperm by other cell types with different densities (data not shown). This was also confirmed by phase-contrast microscopy.

After disruption of the washed sperm by nitrogen cavitation and removal of the cellular debris by differential centrifugation, the plasma membrane was isolated by sucrose-gradient centrifugation. As judged by turbidity, a single membrane band was found which corresponded to a density of 1.13 ± 0.01 g/cm^3 ($n = 6$) (data not shown). These densities are similar to those reported for plasma membranes from boar and bull [16,37,38]. No turbidity was observed at densities of 1.17 to 1.19 g/cm^3 which could correspond to either fused

plasma-acrosomal membranes, acrosomal membranes, or cytoplasmic droplets [16,37,38]. Plasma membranes from epididymal sperm of the caput, corpus and cauda had the same densities, indicating that the densities of sperm plasma membranes did not change during epididymal maturation.

The partially purified membranes were assayed for marker enzyme activities typically associated with plasma or other membranes of sperm. As shown in Table I, the partially purified membranes from boar epididymal sperm were enriched in all enzymatic activities that are usually associated with plasma membranes [15,16,38]. For instance, the specific activity for the alkaline phosphatase was 3–16-times higher in the sperm plasma membrane, relative to the homogenate from any of the three sections of the epididymis. This enrichment in alkaline phosphatase in the plasma membrane is similar to that reported previously by us [15] and others [16,38]. Similarly, the membranes from sperm of the caput, corpus and cauda were enriched at least 4-fold for acid phosphatase and 5′-nucleotidase and 2–11-fold for $(Na^+ + K^+)$-ATPase. However, there was no enrichment in activity of the mitochondrial marker, succinic acid dehydrogenase and, in fact, the specific activities declined during purification of the membranes. Similarly, the specific activities for the acrosomal markers, *N*-acetylglucosaminidase and hyaluronidase, decreased during membrane purification, indicating minimal acrosomal contamination. In addition, the sperm membranes from the three sections of the epididymis contained no DNA or RNA (Table II). The above results on the density and enzymatic activities of the membrane preparations suggested that we purified the plasma membranes of sperm from three different sections of the boar epididymis which had minimal contamination from non-membranous macromolecules such as DNA or RNA or from other sperm membranes such as acrosomal, mitochondrial, or nuclear.

The composition of the purified plasma membranes of boar sperm from the three sections of the epididymis is shown in Table II. Epididymal sperm membrane preparations from the corpus had the lowest amounts of protein, whereas membranes from caput and cauda sperm were similar. The three membrane preparations had different amounts of each individual lipid class. Phospholipids were the major lipid class of the plasma membrane, consisting of about 70% of the total membrane lipid. Membranes of sperm from the caput or cauda of the epididymis had the highest phospholipid content compared to membranes of sperm from the corpus which had the lowest. However, the phospholipid/protein ratio remained constant within experimental error. In the starting suspension of disrupted sperm, the phospholipid/protein ratio was 0.2–0.25 for all three

TABLE I

SPECIFIC ACTIVITIES OF ENZYMES IN MEMBRANE FRACTIONS

Numbers represent nmol/min per mg protein. Each value is mean ± S.E. of four determinations (two preparations). The assays were conducted as described in Materials and Methods. Homogenate is the sperm suspension following disruption by nitrogen cavitation.

Enzymes	Caput			Corpus			Cauda		
	Homogenate	Membrane	Enrichment	Homogenate	Membrane	Enrichment	Homogenate	Membrane	Enrichment
Alkaline phosphatase	19.0 ± 1.8	303.0 ± 4.8	16	13.5 ± 1.3	124.5 ± 6.5	9	69.8 ± 3.6	232.9 ± 23.5	3
Acid phosphatase	4.8 ± 0.8	54.8 ± 0.9	11	10.5 ± 0.8	193.3 ± 22.9	18	14.3 ± 0.3	51.6 ± 9.0	4
5′-Nucleotidase	10.7 ± 0.7	74.9 ± 9.5	7	2.4 ± 0.5	38.5 ± 4.3	6	5.5 ± 0.7	35.8 ± 3.8	6
$(Na^+ + K^+)$-ATPase	2.6 ± 0.1	4.9 ± 0.3	2	4.3 ± 1.6	11.2 ± 3.0	3	0.6 ± 0.1	6.4 ± 1.7	11
Succinate dehydrogenase	10.0 ± 4.0	0.2 ± 0.2	0.02	7.0 ± 1.5	0.4 ± 0.4	0.06	30.0 ± 0.4	0.5 ± 0.3	0.02
N-Acetylglucosaminidase	14.3 ± 0.6	4.8 ± 0.9	0.3	11.4 ± 0.5	3.6 ± 1.1	0.3	12.1 ± 1.0	1.3 ± 0.7	1
Hyaluronidase	4.6 ± 0.7	0.7 ± 0.1	0.2	4.3 ± 0.5	0.8 ± 0.2	0.2	6.6 ± 0.3	1.6 ± 0.1	0.2

TABLE II

BIOCHEMICAL COMPOSITION OF PLASMA MEMBRANES

Values are the total nmol (or mg where indicated) from membranes isolated from 10^9 sperm as described in the text. Each value is the mean ± S.E., $n = 4$.

Component	Composition per 10^9 sperm		
	Caput	Corpus	Cauda
Protein (mg)	0.35 ± 0.07	0.22 ± 0.05	0.34 ± 0.05
Phospholipid (mg) [a]	0.23 ± 0.05	0.15 ± 0.03	0.23 ± 0.04
Phospholipid/protein [a]	0.66 ± 0.06	0.68 ± 0.04	0.68 ± 0.05
DNA (mg)	0	0	0
RNA (mg)	0	0	0
Lipid classes after TLC [b]			
Phospholipid	285.0 ±14.7(73.2)	207.9 ± 9.6(70.2)	276.1 ±12.7(76.2)
Sterols	51.4 ± 6.6(13.2)	43.1 ± 4.9(14.6)	46.2 ± 4.5(12.7)
Glycolipid	38.3 ± 4.3 (9.8)	26.9 ± 5.0 (9.1)	19.6 ± 3.0 (5.4)
Free fatty acid	11.7 ± 0.6 (3.0)	13.6 ± 2.5 (4.6)	1.9 ± 0.1 (0.5)
Diacylglycerols	2.9 ± 0.7 (0.7)	4.5 ± 0.9 (1.5)	18.6 ± 2.7 (5.1)
Triacylglycerols	trace	trace	trace
Cholesteryl esters	0	0	0
Total	389.3 ±26.4	296.0 ±23.7	362.4 ±25.2
Total sterol/ phospholipid [a]	0.18 ± 0.05	0.21 ± 0.04	0.17 ± 0.04
Cholesterol/ phospholipid [a]	0.16 ± 0.05	0.16 ± 0.05	0.12 ± 0.04

[a] A factor of 650 mg/mmol was used for the conversion of μmol of lipid phosphate to mg of phospholipid. This factor is the weighted average of the molecular weights of the individual phospholipids in boar sperm plasma membranes as determined according to their relative abundance. The ratios for phospholipid/protein are on a weight basis, whereas those for sterol and cholesterol are mol/mol. Cholesterol values are from Table VII.

[b] Lipids were fractionated on silica gel H plates as described in Materials and Methods. The amount of each component was determined by phosphate assay for phospholipids or GLC for the other lipids. Numbers in parentheses represent mol% lipid composition.

sections of the epididymis (data not shown). Therefore, the ratio of about 0.7 after membrane purification, shows about a 3-fold increase as expected for membrane isolation. The phospholipid composition of the plasma membrane will be described below.

Sterols were the second major class of lipids in the sperm plasma membranes and their amount remained constant within experimental error during epididymal transit. It should also be noted that the cholesterol/phospholipid and total sterol/phospholipid ratios of the isolated plasma membranes from the three sections of the epididymis remain constant within experimental error. There was one major glycolipid in sperm plasma membranes. Its composition will be discussed in more detail below, but it has been tentatively identified as a 3′-*O*-sulfomonogalactosyldiacylglycerol. As shown in Table II, the glycolipid content of the membranes decreased by about 50% during epididymal transit from the caput to the cauda.

Sperm plasma membrane from the caput and corpus of the epididymis contained free fatty acids equivalent to 2–3% of the phospholipid esterified fatty acids, while caudal sperm plasma membrane contained only traces of free fatty acid. The total amount of membrane diacylglycerols increased 6-times as the sperm migrated from the caput to the cauda of the epididymis. Finally, there were little or no detectable triacylglycerols and cholesteryl fatty acid esters.

The phospholipid composition of isolated

plasma membrane of sperm from the caput, corpus and cauda of the epididymis is shown in Table III. PC was the major phospholipid of the membranes and the total amount increased from caput to cauda. In the subclasses of PC, the diacyl species represented only about 25 to 30% of the total PC, while about 50% of the PC was alkylacyl and the remainder alk-1-enylacyl (plasmalogen) PC. During transit through the epididymis, the diacylPC was almost constant, while the alkylacyl and alkenylacyl classes increased. The second major phospholipid was PE and the total amount in plasma membranes decreased during epididymal transit of the sperm so that the PE/PC ratio decreased from 1.1 in caput to 0.8 in corpus and 0.7 in cauda. In the subclasses of PE, the diacyl species was less than 30% of the total PE and decreased during transit through the epididymis. In contrast to PC, the alk-1-enylacyl species was the major PE component and this decreased in amount while the alkylacyl species was almost constant in the plasma membranes at different points of transit through the epididymis.

TABLE III

PLASMA MEMBRANE PHOSPHOLIPID COMPOSITION

The lipid extracts of sperm plasma membranes were separated by two-dimensional TLC and quantitated by phosphate assay of the eluted spots. The subclasses of PC and PE were determined by hydrolysis with phospholipase C and further TLC and GLC as described in Materials and Methods. In the case of diacyl species, the values listed were calculated from the amounts of fatty acid measured by GLC. Results are expressed as means ± S.E., $n = 4$. Sph, sphingomyelin; LPC, lysophosphatidylcholine.

Phospholipid	Composition (mol%)		
	Caput	Corpus	Cauda
Total PC	32.5 ±0.2	41.2±0.5	39.9±0.2
DiacylPC	10.3 ±0.1	10.0±0.1	9.2±0.1
AlkylacylPC	16.7 ±0.1	24.0±0.1	22.4±0.1
AlkenylacylPC	5.5 ±0.1	7.2±0.1	8.3±0.1
Total PE	35.2 ±0.2	32.2±0.4	27.7±0.1
DiacylPE	11.5 ±0.1	7.5±0.1	6.7±0.1
AlkylacylPE	7.8 ±0.2	10.5±0.1	9.5±0.2
AlkenylacylPE	15.9 ±0.1	14.2±0.1	11.5±0.2
Sph	17.7 ±0.3	15.9±0.6	23.0±0.4
PS	6.7 ±0.2	3.8±0.5	3.3±0.1
PI	5.6 ±0.3	5.3±0.5	2.9±0.1
PIP	0.06±0.03	0.7±0.1	0.5±0.1
PIP_2	0.1 ±0.05	0.5±0.1	0.6±0.02
LPC	2.2 ±0.2	0.4±0.2	2.1±0.3

The amount of sphingomyelin increased in the sperm plasma membranes from caput to cauda so that finally the relative amounts of the choline phospholipids increased by 13%. Lysophosphatidylcholine was present in minor amounts, comprising 2% or less of the total phospholipid.

The acidic phospholipids, PS and PI each accounted for 3–7% of the total phospholipid and their relative amounts decreased by about 50% during transit of sperm from caput to cauda. On the contrary, the relative amounts of two minor membrane constituents, PIP and PIP_2, increased 8- and 6-fold, respectively, in membranes from cauda vs. caput sperm.

The major fatty acids of boar sperm plasma membranes were 16 : 0 and 22 : 5 (Table IV). During transit of sperm in the epididymis, the major changes were a decrease in the amount of 18 : 1 and 20 : 4 accompanied by an increase in the 22 : 5 and 22 : 6 content. Other fatty acids and the ratio of saturated to unsaturated fatty acids remained relatively constant. Since the major lipid class was phospholipids, we analyzed the fatty acid composition of the five major phospholipids in the sperm plasma membrane and the results are shown in Tables IV and V. Four fatty acids (16 : 0, 18 : 1, 22 : 5 and 22 : 6) comprised approx. 90% of the fatty acids in PC of sperm plasma membrane from the three sections of epididymis. During epididymal maturation the amount of 16 : 0, 18 : 1 and 20 : 4 decreased, whereas the amount of 18 : 0, 22 : 5 and 22 : 6 increased. PE contained the same fatty acids as PC except for 20 : 3 and it contained higher amounts of saturated fatty acids, mainly palmitic acid. The changes in the fatty acid composition of the plasma membrane PE during epididymal maturation can be summarized as a decrease in the percentage of 16 : 0, 18 : 1 and 20 : 4 and an increase in the percentage of 22 : 5 and 22 : 6 (Table IV).

Sphingomyelin was unique in that saturated fatty acids comprised about 97% of the fatty acids (Table V). The most significant change in the fatty acid composition of sphingomyelin was a decrease in the amount of 16 : 0 accompanied by an increase in the amount of 14 : 0 and 20 : 0 during transit from the caput to the cauda. PS was notable for its high 18 : 0 content and an increase in

TABLE IV

FATTY ACID COMPOSITION OF MEMBRANE LIPIDS

Sperm plasma membrane lipids were fractionated, transesterified and analyzed by GLC as described in Materials and Methods. Numbers present mol%. Each value is the mean ± S.E., $n = 4$, n.d., not detectable.

Fatty acids	Total fatty acid composition			PC composition			PE composition		
	Caput	Corpus	Cauda	Caput	Corpus	Cauda	Caput	Corpus	Cauda
14:0	6.4±0.6	3.3±0.9	7.5±1.1	1.2±0.1	2.8±0.3	4.3±0.3	0.4±0.1	1.0±0.2	0.9±0.1
16:0	49.1±3.2	54.2±5.4	44.7±4.0	39.1±1.5	48.7±1.8	30.1±3.2	61.2±4.6	56.9±4.6	45.8±4.3
18:0	7.1±0.7	7.5±1.0	8.5±0.7	2.7±0.3	3.0±0.4	5.0±0.6	4.3±0.9	5.1±0.6	4.0±0.3
18:1	9.9±0.8	7.0±1.1	3.1±0.6	20.7±2.6	8.7±0.7	4.0±0.3	6.3±1.0	3.5±0.3	3.0±1.0
18:2	1.2±0.3	2.0±0.3	0.7±0.1	1.9±0.4	3.5±0.6	0.6±0.1	0.8±0.1	0.8±0.2	0.3±0.1
18:3	n.d.	n.d.	0.5±0.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20:3	0.6±0.2	0.7±0.3	0.7±0.1	1.3±0.2	1.2±0.1	1.1±0.1	n.d.	n.d.	n.d.
20:4	2.7±0.4	1.7±0.2	1.4±0.3	2.1±0.1	1.5±0.2	n.d.	2.4±0.4	2.4±0.3	1.1±0.1
22:4	0.9±0.2	1.9±0.6	1.0±0.1	0.9±0.1	1.2±0.1	1.2±0.2	0.8±0.1	1.2±0.3	1.4±0.1
22:5	16.4±2.1	15.9±3.1	23.4±1.6	23.7±2.0	22.7±1.1	38.9±1.9	18.5±3.0	23.6±4.1	33.2±2.0
22:6	5.6±2.3	5.7±1.5	8.5±0.5	6.4±1.5	6.6±0.6	14.8±2.9	5.3±1.1	5.6±1.5	10.3±1.7
% Satd.	62.6±4.5	65.0±7.3	60.7±5.8	43.0±4.3	54.5±3.1	39.4±4.7	65.9±5.6	63.0±6.0	50.7±4.8

arachidonic acid content in the sperm membranes from caput to corpus to cauda, whereas in all other phospholipids the content of 20:4 decreased. The changes in the content of the other fatty acids were similar but not identical to those in the major phospholipids. For PI, 18:0 was again a major fatty acid and, together with 16:0, comprised over 90% of the fatty acids. During transit through the epididymis, there were reciprocal changes in the content of 16:0 and 18:0 in sperm plasma membrane PI. It is possible that changes in the fatty composition of the minor (less than 2%) phospholipids such as PIP, PIP_2 and lysophosphatidylcholine might occur, but our methods were not sensitive enough to detect such changes.

TABLE V

FATTY ACID COMPOSITION OF MEMBRANE LIPIDS

Sperm plasma membrane lipids were fractionated, transesterified and analyzed by GLC as described in Materials and Methods. Numbers represent mol%. Each value is the mean ± S.E., $n = 4$. Sph, sphingomyelin.

Fatty acids	Sph composition			PS composition			PI composition		
	Caput	Corpus	Cauda	Caput	Corpus	Cauda	Caput	Corpus	Cauda
14:0	1.9±0.2	4.3±1.0	6.1±0.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
16:0	66.9±3.7	65.2±2.4	53.0±3.4	37.4±4.0	26.7±4.8	23.3±1.4	55.6±1.7	52.6±2.1	49.9±1.5
18:0	12.6±1.5	6.8±1.1	12.1±0.9	32.4±2.5	34.8±2.3	40.9±2.3	36.8±1.0	38.2±1.1	48.2±1.5
18:1	1.5±0.2	3.3±0.3	2.9±0.5	9.2±1.1	9.8±0.5	6.0±0.1	3.6±0.3	3.8±0.5	0.9±0.4
18:2	n.d.	n.d.	n.d.	n.d.	0.4±0.1	2.6±0.3	n.d.	n.d.	n.d.
20:0	12.9±0.7	17.3±2.8	23.2±4.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20:3	n.d.	n.d.	n.d.	3.8±0.7	3.4±0.3	n.d.	n.d.	n.d.	n.d.
20:4	n.d.	n.d.	n.d.	4.6±1.2	7.5±0.7	8.8±1.1	4.0±1.0	5.4±0.6	1.0±0.6
22:0	4.1±0.3	3.1±0.8	2.8±0.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22:5	n.d.	n.d.	n.d.	12.6±2.1	17.4±3.8	18.4±3.0	n.d.	n.d.	n.d.
% Satd.	98.4±6.4	96.7±8.4	97.2±10.9	69.8±6.5	61.5±7.1	64.2±3.7	92.4±2.7	90.8±3.2	98.1±3.0

In addition to the major phospholipids, we also determined the composition of the free fatty acid pool and the diacylglycerols (Table VI). In both lipids, the unusually high amounts of the total saturated fatty acid, specifically 14:0, increased during transit of sperm in the epididymis.

The boar sperm plasma membrane contained two sterols which were identified by GLC–mass spectrometry as cholesterol and desmosterol. As shown in Table VII, there was a decrease in the amount of cholesterol accompanied by an increase in the amount of desmosterol during epididymal maturation of boar sperm so that the desmosterol/cholesterol ratio changed from 0.14 in the caput to 0.27 in the corpus to 0.33 in the cauda. We also measured the amount of sterol sulfate, as described in Materials and Methods, which showed that the only sulfated sterol was cholesterol sulfate. Caput and corpus sperm plasma membrane contained the same amount, within experimental error, whereas caudal membrane contained higher amounts of the sulfated sterol.

TABLE VI

FATTY ACID COMPOSITION OF MEMBRANE FREE FATTY ACIDS AND DIACYLGLYCEROL

Free fatty acids and diacylglycerols were isolated, transesterified and analyzed by GLC as described in Materials and Methods. Results are expressed as the mean ± S.E., $n = 4$. n.d., not detectable.

Free fatty acids	Composition (mol%)		
	Caput	Corpus	Cauda
14:0	3.2±0.3	6.5±0.3	29.3±1.0
16:0	56.0±1.8	62.8±4.9	43.8±0.9
18:0	19.8±0.8	13.6±0.5	14.9±0.7
18:1	15.8±1.3	12.6±1.9	10.2±0.8
18:2	1.5±0.2	3.6±1.5	1.8±0.1
20:4	2.5±0.5	n.d.	n.d.
22:0	1.2±0.2	0.8±0.2	n.d.
% Satd.	80.2±3.1	83.7±5.9	88.0±2.6
Diacylglycerol fatty acids			
14:0	18.9±0.5	35.8±4.1	72.4±2.9
16:0	42.3±0.5	42.1±1.2	20.1±1.6
18:0	25.9±1.1	13.1±1.5	5.4±0.8
18:1	11.9±1.2	8.4±1.3	1.8±0.4
18:2	0.9±0.3	0.7±0.1	0.3±0.1
% Satd.	87.1±2.1	91.0±6.8	97.9±5.3

TABLE VII

STEROL COMPOSITION OF PLASMA MEMBRANE

The amount of each sterol was determined after TLC separation of the lipid extract, elution of the sterols from the plate and GLC. Results are expressed as means ± S.E., $n = 4$.

Sterol	Composition (nmol/10^9 sperm)		
	Caput	Corpus	Cauda
Cholesterol	44.5 ±5.4	33.5 ±2.9	33.7 ±2.2
Desmosterol	6.1 ±1.0	9.0 ±2.0	11.1 ±2.3
Cholesterol sulfate	0.8 ±0.2	0.6 ±0.2	1.4 ±0.2
Total sterol	51.4 ±6.6	43.1 ±4.9	46.2 ±4.5
Desmosterol/ cholesterol	0.14±0.04	0.27±0.03	0.33±0.05

One major glycolipid was isolated by silicic acid chromatography as described in Materials and Methods. Another glycolipid was also present but in minor quantities which did not permit further characterization. The major glycolipid was molybdate and orcinol negative, positive for lipid-bound sulfate and it comigrated on TLC with commercial monogalactosyldiacylglycerol after mild methanolysis which removed the sulfate group [9]. Further analysis by hydrolysis in acidified methanol, differential extraction of the methanolysis products, TLC and GLC of the extracts, as described in Material and Methods, revealed that the glycolipid contained: (a) a sugar identified as galactose, (b) fatty acids and (c) glyceryl ether identified as 1-*O*-hexadecyl-DL-glycerol by GLC.

TABLE VIII

FATTY ACID COMPOSITION OF MONOGALACTOSYLDIACYLGLYCEROL SULFATE

The glycolipid was isolated, the fatty acids were transesterified and the methyl esters were analyzed by GLC as described in Materials and Methods. Results are expressed as means ± S.E., $n = 4$.

Fatty acid	Composition (mol%)		
	Caput	Corpus	Cauda
14:0	4.0±0.5	6.1±1.1	2.7±0.2
16:0	92.5±0.3	88.8±2.0	91.7±0.3
18:0	3.4±0.2	5.0±1.0	4.2±0.5
18:1	0	0	1.5±0.1

The glyceryl ether/fatty acid/sugar/sulfate ratio was 1:1:0.9:0.8, indicating that the glycolipid was monogalactosyldiacylglycerol sulfate, a glycolipid commonly found in sperm from various mammalian species [28,39]. The fatty acid composition of the glycolipid is shown in Table VIII. The major esterified fatty acid was palmitic acid, while myristic and stearic acid each contributed less than 6% of the total fatty acid in the glycolipid.

Discussion

In this study, sperm plasma membranes from three different sections of the boar epididymis have been isolated. The isolated membranes banded as a single band in a linear sucrose-density gradient used in the final purification step. The three membrane preparations had the same densities, characterisitc of plasma membrane [16,37,38, 40,41] and they were enriched for the commonly used plasma membrane enzyme markers, such as acid and alkaline phosphatases [15,16,38,40,41] $(Na^+ + K^+)$-ATPase [38,42] and 5'-nucleotidase [16,43]. The absence of significant succinic acid dehydrogenase activity in the isolated plasma membrane indicated that there was little if any mitochondrial membrane contamination in this fraction. *N*-Acetylglucosaminidase was assayed because of the reported high activity of this enzyme in both the acrosome [34] and cytoplasmic droplets [44]. Another acrosome marker enzyme is hyaluronidase [32], No enrichment for these enzymatic activities was observed in sperm membranes, indicating minimal contamination from cytoplasmic droplets and acrosomes. it might be noted that the decrease in the specific activity of hyaluronidase, *N*-acetylglucosaminidase and succinic acid dehydrogenase cannot be attributed to inactivation of the enzymes during membrane preparation, since most of the activity was found in the low-speed pellets (data not shown). The fact that these membrane preparations were not free of the first two enzymes could either mean some contamination by acrosomal membranes or that plasma membranes actually have both activities but at reduced levels compared to the acrosome. Some support for the latter possibility comes from reports by Noland et al. [38] which show the presence of *N*-acetylglucosaminidase in sperm plasma membranes.

A striking characteristic of the boar sperm plasma membrane is the observation that the phospholipid/protein and cholesterol/phospholipid ratio remain constant within experimental error during epididymal maturation. Both ratios are commonly correlated with the membrane fluidity which has been shown to change during transit of sperm through the epididymis [13]. Changes in the fatty acid composition of the membrane as well as in the amount of the individual sperm sterols may account for the observed change in fluidity. The changes in the individual sterol amount were an increase in desmosterol and cholesterol sulfate levels and a decrease in the cholesterol level during transit through the epididymis. The occurrence of desmosterol in whole sperm [45] and in sperm plasma membrane, reported by us for the first time, is noteworthy, since ti is an uncommon component of mammalian cells. However, its influence on the membrane properties is uncertain. Low levels of the Δ^{24} reductase, the presence of an inhibitor or transfer from another sperm membrane or the epididymal fluid may account for the increased level of desmosterol in the sperm plasma membrane.

The decrease in the amount of cholesterol of boar sperm plasma membranes is in agreement with previous reports showing diminution of the total sperm cholesterol in hamster [46], rat [48] and ram [12] whole sperm. Sulfo-conjugated sterols have also been detected in hamster epididymal sperm [48] and human ejaculated sperm [49]. The appearance of cholesterol sulfate in the sperm plasma membrane during epididymal maturation may be an important developmental change. Cholesterol sulfate has been reported to stabilize certain membranes, like the erythrocyte plasma membrane [50], and it is known to inhibit in vitro capacitation of hamster sperm [51] and the acrosin reaction [52]. In addition, the existence of sulfatases that can hydrolyze the sterol sulfates has been reported in the female reproductive tract of mammals [50,53]. The above observations might suggest a role for sterol sulfates that could involve regulating the fluidity of the sperm membrane during epididymal maturation or capacitation and the acrosome reaction.

We separated and characterized eight phospholipid fractions in the sperm plasma membrane and also identified and quantitated the alkylacyl and alkenylacyl species in both choline and ethanolamine phosphoglycerides. As observed previously for the whole boar sperm [4], choline and ethanolamine phospholipids were by far the major phospholipids, amounting to about 70% of the total lipid phosphorus. An increase in the PC accompanied by a decrease in the PE was observed during epididymal maturation of the sperm. About 2/3 of the choline and ethanolamine phosphoglycerides were found to contain ether linkages. Although during the past few years the properties of ether lipids have attracted the interest of biochemists [54], the specific function of these lipids in biological membranes is still not understood. They could effect the membrane properties by limiting the availability of substrate for phospholipase A_2, since plasmalogens and alkylacyl phospholipids exhibit different rates of hydrolysis than their diacyl counterparts [55,56]. Alternatively, they could provide the substrate for a specific enzyme to produce specific lysophospholipids [57] or they might act as precursors for the biosynthesis of biologically active compounds, e.g., the platelet-activating factor, 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine [58].

The phosphatidylinositol content of the plasma membrane decreased 2-fold during epididymal maturation of boar sperm, whereas the amount of polyphosphoinositides increased 6–8-fold. Polyphosphoinositides appear to be ubiquitous components of eucaryotic cells of mammals, birds, fish, crustacea, insects, green plants, fungi and protists. It has been suggested that polyphosphoinositides might be characteristic lipids of plasma membranes [59,60]. Polyphosphoinositides are minor lipid components, but this may conceal the fact that they are present at quite high local concentrations at the inner surface of the plasma membrane. Recently, the hydrolysis of polyphosphoinositides has been linked with the activation of receptors that use calcium as a second messenger [61].

The fatty acid composition of the total membrane lipid as well as that of the major phosphoglycerides was found to be dominated by 16 : 0 and 22 : 5 as the major saturated and unsaturated species, respectively, although 18 : 1 and 22 : 6 were also present in relatively high amounts. These results are qualitatively close to those reported for the whole boar epididymal sperm phospholipid composition [3,62,63]. During epididymal maturation, the degree of fatty acid unsaturation in the total membrane lipid did not change. The only change observed was an increase in the 22 : 5 and 22 : 6 content together with a compensatory decrease in the 18 : 1 and 20 : 4 amount. The increase in 22 : 5 may be significant, since it has been shown to be the elongation and desaturation product of arachidonic acid in rat testis [64]. An increase in the amount of this fatty acid has been observed in rat testis during sexual maturation [65] and the increased levels were correlated with maturation of the spermatids [66]. Other experiments also showed a conversoin of [^{14}C]22 : 5 to arachidonic acid in rat testis [67]. The other highly unsaturated fatty acid of epididymal sperm plasma membrane, 22 : 6, has been shown to convert to 22 : 5 and finally to 20 : 5, a fatty acid with unusual properties [68], during catabolism in rat testis [69].

Both 22 : 5 and 22 : 6 fatty acids contain methylene-interrupted double bonds. Studies [70] with 18 : 0/22 : 6-PC indicated that the double bonds affected the conformation of the phospholipid in the bilayer by limiting the motional freedom due to the lack of rotation at the six double bonds and by decreasing the effective chain length. Moreover, polyunsaturation affects the phospholipid interaction with other membrane components. Whereas cholesterol has a condensing effect on phosphoglycerides esterified with a saturated and an unsaturated fatty acyl chain, there is only a small cholesterol condensing effect with 16 : 0/22 : 6-PC (for review, see Ref. 70).

The major glycolipid of the boar sperm plasma membrane, from the caput, corpus and cauda of the epididymis, has been found to be monoalkylmonoacylglyceryl monogalactoside sulfate. A similar compound is present in the testes of rat, mouse, guinea pig, rabbit, boar and man, in epididymal bovine sperm and ejaculated boar sperm [9,28,39]; however, this is the first report of the existence of the glycolipid in sperm plasma membrane. The only other mammalian tissue where glyceroglycolipids have been found so far is nervous tissue. Evidence exists that sulfogalactolipid can be synthesized in vitro from galactog-

lycerolipid by the action of a Golgi-enriched sulfotransferase, using phosphoadenosine phosphosulfate as the sulfate donor [71].

Finally, we observed changes in the fatty acid and diacylglycerol levels of the plasma membrane during epididymal maturation of the boar sperm. Fatty acids and diacylglycerols are amphipathic molecules ideally suited for membranes and are commonly found in all membranes analyzed. Berridge [61] reported recently that diacylglycerol has many of the properties of a classical second messenger. It is produced very rapidly, acts at very low concentrations, and there are specific mechanisms for removing the messenger once the internal signal is withdrawn. For instance, diacylglycerol has been proposed to act as a second messenger to activate protein kinase C which is an ubiquitous enzyme present in all organs examined [72,73]. When activated by diacylglycerol, protein kinase C phosphorylates specific cellular proteins which contribute to various physiological processes, particularly secretion and proliferation. The fatty acid esterified to the diacylglycerol were saturated which is in agreement with previous reports on boar [4] and bull whole sperm [10].

Summarizing this study we have (a) isolated sperm from three sections of the boar epididymis and partially purified the plasma membrane from these sperm, (b) characterized the plasma membranes of sperm from caput, corpus and cauda epididymis with respect to enzymatic activities, protein, DNA, RNA, phospholipid and sterol content, (c) analyzed the lipid classes of the purified membranes and quantitated changes occurring during epididymal maturation. Since this is the first report on sperm plasma membrane lipid changes during epididymal maturation, except for the work done on ram sperm plasma membrane by Parks and Hammerstedt (unpublished observations), further studies are needed to establish the metabolic pathways by which these changes take place, as well as their physiological significances.

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