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Lipid changes of goat sperm plasma membrane during epididymal maturation

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Highly purified plasma membranes of maturing goat caput-, corpus- and cauda-epididymal spermatozoa were isolated by aqueous two-phase polymer methods and their lipid constituents were analysed. Phospholipid (approx. 75% w/w), neutral lipid (approx. 15% w/w) and glycolipid (approx. 10% w/w) were the major sperm membrane lipids. There was a significant decrease in the total lipids (approx. 25% w/w), phospholipid (approx. 30% w/w) and glycolipid (approx. 80% w/w) contents of sperm membrane during epididymal maturation. On the contrary, the mature cauda-sperm membrane showed greater (approx. 50% w/w) neutral lipid content than that of the immature caput sperm. Phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin were the phospholipids of the sperm membrane, the former two being the major lipids. Both PC and PE fractions consisted of three species – diacyl, alkylacyl and alkenylacyl forms, the last one being the dominant species in both PC and PE. Of all the phospholipids, diacyl PE decreased most strikingly (approx. 65% w/w) during sperm maturation. The neutral lipid fraction contained sterols, wax esters, 1-*O*-alkyl-2,3-diacylglycerol, triacylglycerol and fatty acids. Sterols represented nearly 75% w/w of the neutral lipids and cholesterol was the major component (approx. 95% w/w) of the sterol fraction. The sperm maturity was associated with marked increase of sterol (approx. 60% w/w) and steryl ester (approx. 200% w/w) and decrease (approx. 50–65% w/w) of the other membrane-bound neutral lipids. The glycolipid was identified as monogalactosyldiacylglycerol. The fatty acid profile of the various membrane lipids underwent marked alteration during the epididymal transit of the male gametes. Cholesterol/phospholipid and saturated/unsaturated fatty acid ratios increased greatly in the maturing sperm membrane. The altered lipid profile of the mature sperm membrane leads to changes in its fluidity that play an important role in determining the structure and functions of the biomembrane.

Introduction

Cell surface molecules regulate the functions of the mammalian cells by modulating cell–cell interactions, effector–receptor interactions, membrane permeability, transmembrane signalling, etc. [1]. The spermatozoal surface has been implicated as playing a vital role during fertilisation and maturation, when the immature testicular spermatozoa acquire forward progression and fertility as the male gametes traverse the epididymis [2,3]. Ultrastructural studies have shown that the appearance and topographic configuration of sperm plasma membrane are altered during the epididymal maturation [4]. A variety of maturation-dependent biochemical changes of the sperm membranes have been reported [2,3,5]. For example, during the epididymal maturity the sperm membrane undergoes marked modifications in respect of antigenic properties [6], glycoproteins [7], lectin receptors [8], ATPase activity [9], thiol groups [10], phosphoproteins, and protein kinase and phosphatase activities [11,12]. The chemical nature of the cell surface molecules that may regulate sperm motility and fertility is not well understood.

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Abbreviations: NL, neutral lipid; PL, phospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; MGDG, monogalactosyldiacylglycerol; Alk-DAG, 1-*O*-alkyl-diacylglycerol; DAG, diacylglycerol; TG, triacylglycerol; GLC, gas-liquid chromatography; TLC, thin-layer chromatography; RRT, relative retention time; FFA, free fatty acids; medium A contains 119 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM glucose, 16.3 mM potassium phosphate (pH 6.9) and penicillin, 50 units/ml.

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Lipid, a major constituent of the plasma membrane, plays an important role in constituting the membrane structure by modulating the fluidity of the biomembranes [13,14]. Consequently, the lipid constituents have a profound influence on the regulation of membrane functions. Reports on changes of sperm lipid composition during epididymal maturation were mostly based on lipid extracts of whole sperm cells [15–18]. A decrease in the content of sperm total lipid during transit of spermatozoa through epididymis has been reported in boar [16], bull [18], ram [19,20] and rat [21]. This decrease in the lipid contents of sperm as a consequence of epididymal maturity may be due to their utilisation as energy source. Because the plasma membrane represents less than 35% of the total cellular lipids [22], the data obtained with whole cell lipid analysis are of nominal use in explaining plasma membrane features in relation to its lipid composition.

Limited data are available on the lipid constituents of the maturing sperm membrane [23,24]. The membranes derived from ram [23] and boar [24] spermatozoa were analysed for gross lipid composition and individual phospholipid contents. Parks and Hammerstedt [23] have estimated the major fatty acids of ram sperm membrane PC and total phospholipids. Nikolopoulou et al. [24], however, have reported more extensive analysis of free fatty acids as well as bound fatty acids of the various lipids of the boar sperm membranes. These investigators have also reported the occurrence of a glycolipid and diacyl, alkylacyl and alkenylacyl froms of PC and PE in the boar membrane. Although both the species showed marked changes in their membrane lipids during epididymal sperm maturation, some differences were observed in the nature of the changes. For example, the sperm maturity was associated with a marked increase of cholesterol/phospholipid ratio in ram sperm as opposed to boar sperm, where this ratio decreased significantly [23,24].

In the present investigation, highly purified plasma membranes were isolated from goat cuput-, corpus- and cauda-epididymal spermatozoa and their lipid composition and fatty acids have been analysed in detail to establish the possible functions of membrane lipids in epididymal maturation of spermatozoa.

Materials and Methods

Material. Dextran (average molecular weight, 229000), poly(ethylene glycol) compound (molecular weight range 15000–20000) and lipid standards were purchased from Sigma, St. Louis, MO, U.S.A. Solid supports and liquid phases for gas-liquid chromatography (GLC) columns were obtained from Pierce Chemical, Rockford, IL, U.S.A. All the solvents used were analytical reagents and were purified and distilled, whenever necessary. Silica gel G and H for thin-layer chromatography (TLC) were obtained from Merck, Darmstadt, F.R.G. Silicic acid used for column chromatography was unisil (100–200 mesh) from Clarkson Chemical, Williamsport, PA, U.S.A.

Isolation of maturing goat epididymal spermatozoa. Spermatozoa acquire maturity during passage through caput (first part) and corpus (middle part) portions of the epididymis and the mature spermatozoa are stored in cauda (distal) end of the epididymis. Spermatozoa from the different segments of the epididymis were extracted in medium A [25]. The corpus- and cauda-epididymal spermatozoa were sedimented by low-speed centrifugation and then washed in medium A. The immature caput-epididymal spermatozoa were purified by a discontinuous Ficoll-400 density gradient centrifugation method developed in our laboraotry [26].

Isolation of sperm plasma membrane. Membrane was isolated from the mature cauda-spermatozoa by using an improved aqueous two-phase polymer method [25]. Membranes derived from the caput- and corpus-spermatozoa were isolated by a modification of this two-phase polymer method [27]. The method consists of hypotonic shock of intact spermatozoa with 1.25 mM EDTA to dissociate membrane and dispersion of these cells to a two-phase polymer system consisting of 5.5% dextran and 4.2% polyethylene glycol, prior to centrifugation at $9700 \times g$ (for cauda-spermatozoa) or $12000 \times g$ (for caput- and corpus-sperm) for 30 min when the two polymer phases become separated and the membranes sediment at the interphase. The isolated membranes showed high degree of purity as evidence by electron microscopic studies and analyses of marker enzymes characteristic of cellular organelles. The isolated membranes were dispersed in 10 mM Tris-HCl (pH 7.4). Protein contents of the membrane preparations were estimated according to Lowry et al. [28] using bovine serum albumin as standard.

Lipid extraction. After the isolation of sperm membranes from caput, corpus and cauda epididymides of goat, extraction of lipids were carried out immediately, essentially following the method of Folch et al. [29]. The isolated membranes (100 mg protein) were homogenised for 1 min with 10 ml of methanol, then 20 ml of chloroform were added and the process of homogenisation was continued for further 2 min. The mixture was centrifuged and the solid residue was resuspended in chloroform/methanol (2:1, v/v, 30 ml) and homogenised for 3 min. After centrifugation the solid was washed once more with chloroform (20 ml) and once with methanol (10 ml). The combined supernatants were transferred to a separating funnel, diluted with one quarter of the total volume of 0.88% potassium chloride in water and the mixture was shaken thoroughly and allowed to settle. The lower layer was transferred to another separating funnel and equal volume of water/methanol (1:1, v/v) was added, shaken and allowed to

settle. The upper layer was washed thrice with water/methanol (1 : 1, v/v). The bottom layer contained purified lipid, which was dried over anhydrous sodium sulphate. The solvent was removed in a rotary evaporator at room temperature; finally the lipid was weighed and stored in a deep freezer at −20 °C in redistilled hexane under nitrogen atmosphere. Recovery of the lipid was estimated to be 95 ± 3% based on the recovery of [^{3}H]phosphatidylinositol, which was added to the sample at the beginning of lipid extraction. Unless otherwise stated, lipid contents of the isolated plasma membranes were expressed as μg lipids/mg membrane proteins.

Saponification and separation of hydrolysates. Aliquots of total lipids from different membrane samples were saponified and fatty acids were extracted according to Christie [30]. The lipid sample (100 mg) was hydrolysed by refluxing it with 1 M solution of potassium hydroxide in 95% ethanol (2 ml) for 1 h. Nitrogen gas was bubbled slowly through the mixture during saponification. The solution was cooled, water (5 ml) was added and the solution was extracted thoroughly with diethyl ether (3 × 5 ml) to obtain the non-saponifiables. The aqueous layer was acidified with 6 M hydrochloric acid and extracted with diethyl ether (3 × 5 ml) to obtain free fatty acids. The fatty acids thus obtained were methylated by diazomethane [31].

Fractionation of total lipid into various classes. Total lipids from each of the three samples were fractionated into neutral, glyco- and phospholipid by silicic acid column chromatography essentially according to Rouser et al. [32]. The lipid samples (30–50 mg) in 3–5 ml of chloroform was applied to the top of the column. The neutral, glyco and phospholipid were eluted by 10, 40 and 10 column volumes of chloroform, acetone and methanol, respectively. The solvents were evaporated and weighed, and fractions were kept in redistilled hexane in an atmosphere of nitrogen gas at −20 °C. The phospholipid and glycolipid were also estimated by chemical methods by estimating phosphate [33] for phospholipid and sugar [34] for glycolipid.

Separation of NL components by thin-layer chromatography. The NL were fractionated by preparative TLC using solvent mixture of petroleum ether (40–60 °C)/diethyl ether/acetic acid (90 : 10 : 1, by volume) according to Mangold [35]. Various NL components were identified by comparing their R_F values with those of authentic standards applied in a lane at one side of the TLC plate. The Alk-DAG were further purified by preparative TLC [36] using petroleum ether (40–60 °C)/benzene (95 : 5, v/v).

The esterified NL components thus obtained were quantified by gas-liquid chromatography (GLC) of the fatty acids of each lipid class in the presence of known amounts of methyl pentadecanoate (15 : 0), as an internal standard. Sterols were also quantified by GLC using known amounts of cholestane as internal standard. FFA was directly methylated and analysed by GLC.

Analysis of wax ester. Analysis of fatty acids and alcohols of various wax esters were done by lipolysis using lipid free [37] pancreatic lipase on TLC plate [38] and resulting fatty acids and alcohols were analysed by GLC as methyl esters and acetates, respectively.

The methyl esters were analysed by GLC using polar liquid-phase viz. DEGS (diethylene glycol succinate), which was 10% by weight of solid support (silanised gas chrom-Z, 60–80 mesh). The GLC was performed on a dual-flame, dual-column Pye Unicam Model 104 Gas Chromatograph equipped with dual-flame ionisation detector (FID). During analysis, the oven temperature was 180 °C, while that for detector and injection port were around 240 °C. The carrier gas used was nitrogen, with a flow rate of 60 ml/min. Peaks were identified by (i) using cod liver oil fatty acid methyl ester as secondary standard according to Ackman and Burgher [39]; (ii) semilogarithmic plots [40] of relative retention times (RRT) against carbon chain lengths of the fatty acids of cod liver oil and fitting the logarithm of RRT of the fatty acids under investigation into these plots; (iii) comparison of the chromatograms obtained on hydrogenation [41] of methyl ester samples with those of original samples; and (iv) comparison of the equivalent chain length (ECL) values of the component acids with those in the literature [39,42,43]. The quantification of different components were done by triangulation of peaks and internal normalisation methods.

Alcohols from wax ester were acetylated [44] and analysed by GLC using a 3% OV-17 column by programming the column temperature from 220–300 °C at the rate of 4 C°/min according to Misra et al. [45]. Authentic synthetic standards and previously identified secondary standards were used for identification of the components.

Analysis of sterols and steryl ester. Steryl esters were lipolysed on TLC plate by porcine pancreatic lipase according to the technique of Misra et al. [46]. The sterols and fatty acids produced were separated on the same plate, recovered and fatty acids were methylated and analysed by GLC as described in previous section. Sterols, both free and that obtained by lipolysis, were acetylated [44] and silylated [46] and analysed by GLC on 3% SE-30 and 3% OV-17 columns, respectively. Sterols were identified by comparison of the RRT as reported by Patterson [47] and retention indices as described by Ghosh et al. [48].

Analysis of Alk-DAG. Alkyl diacyl glycerols were initially characterised by strong ester bands at 1735 cm^{-1} and 1180 cm^{-1} and *O*-alkyl-ether band at 1110 cm^{-1} in infrared (IR) spectroscopy. Also, the PMR spectrum revealed the presence of characteristic triplet signal for -CH_2-groups adjacent to ether linkages at δ3.4 ppm. The 1-*O*-alkyl glycerols and methyl esters of

fatty acids were released from the Alk-DAG by transesterification of the latter by refluxing with methanolic hydrogen chloride under anhydrous conditions for 2 h [49]. The product viz. methyl esters and 1-*O*-alkyl glycerols were separated by preparative TLC [35]. The methyl esters were recovered and analysed by GLC as described before. The recovered alkylglycerols were silylated by using Trisil-Z [49] and analysed by GLC on a 3% OV-17 column. Authentic 1-*O*-alkylglycerol standards viz., chimyl(1-*O*-hexadecyl glycerol) and batyl(1-*O*-octadecyl glycerol) alcohols were also silylated and analysed similarly for identification of the peaks.

A portion of 1-*O*-alkyl glycerol was acetylated [44] and was subjected to argentation TLC using 10% silver nitrate [50]. Two bands were recovered, one corresponding to authentic 1-*O*-hexadecyl-2,3-diacetylglycerol (R_F = 0.6) prepared by acetylation of authentic 1-*O*-hexadecylglycerol and the other below it (R_F = 0.45). Portions of both the bands, along with their catalytically reduced [32] products were analysed by GLC. A portion of the compound (R_F = 0.45) was oxidised by permanganate-periodate reagent, fatty acids generated were methylated, purified by TLC and analysed by GLC [41].

Analysis of TG. Triacyl glycerol samples were subjected to alkaline hydrolysis, fatty acids were recovered, methylated and analysed by GLC as described earlier.

Separation and analysis of glycolipid. Glycolipids were separated on preparative TLC plates [51] using silica gel G as adsorbent and chloroform/ methanol/ water (65 : 25 : 4, v/v) as developing solvent. Identifications were made by comparison of R_F values of the components with those of reference compounds. Mono- and digalactosyl diacylglycerols were isolated from wheat flour [52] and were used as reference compounds.

Fatty acid methyl ester of the glycolipid was prepared by transesterification using sodium methoxide in methanol as described by Christie [53], and then analyzed by GLC after adding known amount of methyl pentadecanoate as internal standard. The sugar of the glycolipid was released by hydrolysing with aqueous 1 M HCl acid at 100 °C for 12 h. After cooling, known amount of arabinose (50 μg) was added as internal standard. The reaction mixture was washed three times with hexane and then with chloroform. The aqueous layer was dried by nitrogen at 35 °C and kept overnight in a vacuum desiccator [54]. The residue was treated with 0.8 M $NaBH_4$ in water and the mixture left at room temperature for 2 h. The reaction was stopped by the addition of acetic acid and the solution dried by evaporation with repeated addition of methanol acetic acid (200 : 1, v/v), and left in a vacuum desicator overnight. The alditols thus formed were acetylated by reaction with acetic anhydride at 100 °C for 2 h. Excess reagent was removed by evaporation with addition of toluene. The product in chloroform was washed with water, solvent was removed and derivatives were dissolved in acetone and were analysed by GLC using a 3% OV-17 column at 165 °C. Standard sugars were also derivatised in a similar manner, except hydrolysis by aqueous HCl.

Separation and analysis of PL. Phospholipids were separated on silica gel H plates by two-dimensional technique of Rouser et al. [55] using chloroform/ methanol/25% ammonia (65 : 25 : 5, v/v) in the first direction and chloroform/methanol/acetic acid/water (60 : 80 : 20 : 10 v/v) in the second direction. The plates were exposed to HCl vapour for 4 min after development in the first direction and nitrogen gas was flushed over the plates for 1 h before development in the second direction [56]. The combined diacyl and alkylacylanalogues were separated from the 2-acyllyso forms generated by acid hydrolysis of the plasmalogen. The two forms of PL were extracted from the plates and plasmalogenic forms were estimated by phosphorus determination [33] of the lysoform. The PL contents were calculated by multiplying the value of phosphorous by a factor of 25. The combined diacyl and alkyl acyl forms of PC and PE were dephosphorylated using phospholipase C [57]. The NL formed after phospholipase C digestion were acetylated [44] and separated by TLC using a solvent system of petroleum ether (40–60 °C)/ diethyl ether/acetic acid (90 : 10 : 1 by volume) according to Mangold and Baumann [49]. The separated alkylacylacetyl and diacylacetyl glycerols were recovered and fatty acids were analysed by GLC. Alkylacyl and diacyl forms of PL were quantitated by determining absolute amounts of fatty acids, adding known amount of methyl pentaecanoate to methyl esters of fatty acids.

Results

Components of total lipid, neutral lipid and alkyl chains of Alk-DAG

The gross lipid composition of the sperm membranes as presented in Table I, undergoes significant changes during transit through epididymis. PL represented nearly

TABLE I

Composition of various lipid classes in maturing sperm plasma membranes

The data shown are mean of three experiments that are within ± 10.0% of the mean.

Lipids	Caput	Corpus	Cauda
Total lipids [a]	1.1	0.9	0.8
Neutral lipid [b]	154.0	164.0	221.0
Glycolipid [b]	99.0	43.2	19.2
Phospholipid [b]	847.0	693.0	568.0

[a] Expressed as mg of lipid/mg of membrane protein.
[b] Expressed as μg/mg of membrane protein.

TABLE II

Neutral lipid composition of maturing sperm plasma membranes

The data shown are mean of three experiments, that are within ± 12.0% of the mean.

Neutral lipids [a]	Caput	Corpus	Cauda
Wax ester	5.9	7.4	3.3
Steryl ester	8.9	6.7	24.3
1-*O*-Alkyldiacylglycerol	9.0	7.1	5.3
Triacylglycerol	6.9	3.3	2.2
Fatty acid	11.6	11.0	6.0
Sterol	112.4	128.0	180.0

[a] Expressed as μg/mg of membrane protein.

75% (w/w) of the total lipid in all the three maturing sperm membranes. There was a significant decrease in the total lipid (approx. 25% w/w) and PL (about 30% w/w) contents of membrane during the epididymal maturation of goat spermatozoa. The maturation process was associated with a striking decrease (nearly 80% w/w) in glycolipid contents as well as significant increase (approx. 50% w/w) of neutral lipid of plasma membrane during epididymal maturation.

In Table II, the compositions of various components of NL of the membranes of goat sperm from caput, corpus and cauda regions have been presented. Wax ester, Alk-DAG, TG and free fatty acids of sperm membrane decreased markedly (40–70% w/w), whereas sterol and steryl ester increased by about 60% (w/w) and 200% (w/w), respectively during transit from caput to cauda epididymis.

The identifications of wax ester and Alk-DAG were confirmed by TLC and IR and NMR spectroscopy (unpublished results). Primarily, these compounds were identified tentatively by comparison of their R_F values on TLC with those of authentic standards. IR spectrum showed characteristic strong ester bands at 1735 and 1180 cm^{-1} for both the compounds and a *O*-alkyl ether band at 1110 cm^{-1} for Alk-DAG. PMR (100 MHz) spectrum of the Alk-DAG showed signals at δ5.34 ppm (m, $-C\underline{H}=C\underline{H}-$). 5.18 (m, H-2), 4.24 (AB part of an ABX spectrum with $J_{AB} = 12$ Hz, $J_{Ax} = 6$ Hz, $J_{Bx} = 4$ Hz; H-3), 3.54 (d, $J = 6$ Hz, H-1), 3.4 (t, $J = 6$ Hz, H-1), 2.64 (t, $J = 4$ Hz, $=CH-C\underline{H}_2-CH=$), 2.3 (t, $J = 7$ Hz, $-C\underline{H}_2COO-$), 2.04 (m, $-C\underline{H}_2-CH=CH-$), 1.24 (broad s, $-C\underline{H}_2-$) and 0.88 (t, $J = 6$ Hz, $C\underline{H}_3-CH_2-$), where s, d, t and m indicate singlet, doublet, triplet and multiplet, respectively. The PMR spectrum was in good agreement with those reported by Wood and Snyder [58] and recently, by Pakrashi et al. [59]. On lipase hydrolysis [38], wax esters yielded fatty acids and fatty alcohols which were identified and quantitated by GLC analysis and which were found to be present in 1 : 1 molar ratio. Hydrolysis of Alk-DAG yielded fatty acids and 1-*O*-alkyl glycerols, which were identified and quantitated by GLC analysis and were found to be present in 2 : 1 molar ratio.

Trimethylsilyl derivatives of 1-*O*-alkylglycerols gave three peaks by GLC analysis, two of which were identified as 1-*O*-hexadecyl (chimyl) and 1-*O*-octadecyl (batyl) glycerols, by comparison of retention times with authentic compounds. The unidentified peak preceded to that of 1-*O*-octodecyl glycerol and was partially resolved. Acetylated 1-*O*-alkylglycerols were separated into two bands by argentation TLC, of which the band with $R_F = 0.6$ yielded two peaks by GLC, corresponding to acetyl derivatives of 1-*O*-hexadecyl and 1-*O*-octadecylglycerols. The other band with $R_F = 0.45$ showed IR absorption bands at 3020 and 1650 cm^{-1} indicating the presence of *cis*-double bonds. The catalytically reduced product of this band yielded 1-*O*-octadecyl-2,3-diacetate, having identical retention time on GLC to that of authentic compound prepared by the acetylation of 1-*O*-octadecyl-glycerols (batyl alcohol). Oxidation of this band with permanganate periodate and analysis of the monocarboxylic fatty acid by GLC confirmed it to be a C_9 (9 : 0, nonanoic) acid. All these data confirmed that the compound was 1-*O*-octadec-9′, *cis*-enylglycerol , selachyl alcohol. These confirm that the alkyl groups of the Alk-DAG were of 16 : 0, 18 : 0 and 18 : 1 chain lengths.

A comparison of the GLC retention times of the silyl ether derivatives of the compound in question with those of the authentic ones, confirmed the presence of chimyl (16 : 0), batyl (18 : 0), selachyl (18 : 1) alcohols in the ratio of 2 : 2 : 1. Similar results were obtained in all the maturing sperm membrane lipid fractions with little quantitative variation in the composition of alkyl chain.

Component sterols

Sterol profiles have been shown in Table III. As determined by GLC retention parameter of acetyl and trimethyl-silyl ether derivatives, the maturing sperm

TABLE III

Sterol composition of plasma membranes

The data shown are mean of three experiments that are within ± 15.0% of the mean.

Sterol	Caput [a]	Corpus [a]	Cauda [a]
Unidentified	1.3	3.7	2.0
Unidentified	0.9	1.1	2.0
Cholesterol	104.0	121.0	173.0
Desmosterol	4.7	1.5	1.4
24-Methylene cholesterol	0.9	–	1.4
Stigmasterol	1.1	1.3	1.8
Cholesterol/ phospholipid [b]	0.24	0.34	0.59

[a] Values have been expressed as μg/mg protein.

[b] Ratio of cholesterol to phospholipid is expressed as mol/mol.

membranes contain two unidentified sterol components with low RRT. Cholesterol was the major sterol (approx. 95%) in all the three lipid fractions derived from spermatozoa of caput, corpus and cauda epididymides. The mature cauda-sperm membrane showed much higher cholesterol content than the immature caput-sperm membrane. Desmosterol/cholesterol ratio was found to decrease gradually during sperm maturation. Cholesterol to PL ratio markedly incrased in the sperm membrane during transit from caput to cauda epididymis.

Alcohols from wax ester

Alcohol composition of the three wax ester samples as presented in Table IV, reveals the presence of saturated alcohols with even or odd carbon chains, ranging from C_{18} to C_{30}. Of the alcohols, two were branched chains, viz. 28- and 30-*iso* compounds and the major components in all the maturing sperm samples were with 24-, 28- and 29-carbon chain lengths. Among the major components, the percentages of C_{24} and C_{29} alcohols were found to increase gradually with sperm maturation.

Fatty acid composition of wax ester, steryl ester, Alk-DAG, triacylglycerol, total lipid and free fatty acid

The fatty acid composition of the wax ester, steryl ester, Alk-DAG, triacylglycerol and total lipid of the membranes of sperms obtained from caput, corpus and cauda regions, as presented in Table V, reveals that palmitic acid (16 : 0) was the major component among saturates in all the samples and increased gradually from caput to cauda. The next major saturated fatty acid, stearic acid (18 : 0) occurs in substantial amount in all the lipids except in the total lipid of three membrane preparations. In case of TG, together with 16 : 0, 18 : 0, myristic acid (14 : 0) also occurs in appreciable amounts. Of the monoenoic acids, oleic acid (18 : 1(n − 9)) is the major component in all the samples and it decreased during maturation in all the lipid samples, except in wax ester samples. Among the polyunsaturates, major components present in all the samples were linoleic (18 : 2(n − 6)), arachidonic (20 : 4(n − 6)) and docosahexaenoic (22 : 6(n − 3)) acids. Linolenic acid (18 : 3(n − 3)) was present in substantial amount in all the lipid samples, except in TG samples. In addition to these fatty acids, eicosapentaenoic acid (20 : 5(n − 3)) was present in good amount in wax ester samples. Two isomers of docosapentaenoic (22 : 5) acids were present in appreciable proportion in Alk-DAG samples. All the polyunsaturated fatty acids decreased during maturation, except docosahexaenoic, which has increased. In steryl ester samples, arachidonic acid increased during maturation. Saturated to unsaturated fatty acid ratio increased during maturation, in all the lipid samples, indicating the presence of large proportions of saturates in cauda-sperm membranes, except in steryl ester, where the proportions of saturates is large in corpus-sperm membranes. It is interesting to note that the levels of 22-carbon chain polyunsaturated fatty acids were abnormally high in Alk-DAG with particular reference to docosahexaenoic acid (22 : 6(n − 3)). Fatty acid compositions of FFA were essentially similar to those of total lipid and were not presented separately.

TABLE IV

Composition of alcohols obtained by the lipolysis of wax ester

The data shown are mean of three experiments that are within ± 15.0% of the mean. Alcohols of < 1.0% are listed as 'trace'.

Alcohols [b]	Caput [a]	Corpus [a]	Cauda [a]
18:0	5.2	4.4	–
20:0	3.0	2.4	2.6
21:0	–	trace	–
22:0	5.9	2.2	2.6
23:0	1.8	1.3	1.6
24:0	31.3	27.0	41.7
25:0	–	–	trace
26:0	3.4	2.5	4.9
27:0	–	1.0	1.0
28- *iso*	15.2	–	–
28:0	25.6	39.4	24.6
29:0	8.2	19.1	20.7
30- *iso*	–	trace	–
30:0	trace	trace	–

[a] Values have been expressed as % (w/w) of total alcohols.
[b] Carbon chain length : number of double bonds.

Components of glycolipid and fatty acid composition of MGDG

The only glycolipid present in all the maturing sperm membrane samples was identified as MGDG, by comparing the R_F value on TLC plate, with that of the authentic compound isolated from wheat flour and by the colour reactions of sugar with α-naphthol spray. Only one sugar, viz. galactose was identified by GLC analysis of the trimethylsilyl ether derivatives of the sugars. Molar ratio of fatty acid to sugar was found to be very close to 2.00, thereby confirming the occurrence of MGDG in all the three membrane samples. Fatty acid compositions of MGDG, as obtained by GLC, have been presented in Table VI. Major saturated fatty acids were palmitic (16 : 0) and stearic acids (18 : 0), of which the latter was found to increase during maturation. Among the unsaturated fatty acids, oleic (18 : 1(n − 9)), linoleic (18 : 2(n − 6)), arachidonic (20 : 4(n − 6)), eicosapentaenoic (20 : 5(n − 3)), isomeric docosapentaenoic (22 : 5(n − 6) and 22 : 5(n − 3)) and docosahexaenoic (22 : 6(n − 3)) acids were the major components. Proportions of unsaturates in MGDG decreased in sperm undergoing maturation in the epididymis.

eriments that are within ± 15.0% of the mean for most of the components. Fatty acids of < 1.0% are listed as 'trace'.

			Corpus [b]					Cauda [b]				
Alk-DAG	TG	TL [c]	WE [c]	SE [c]	Alk-DAG	TG	TL [c]	WE [c]	SE [c]	Alk-DAG	TG	TL [c]
3.5	7.8	8.3	21.0	1.2	4.6	10.9	10.7	8.8	3.9	6.4	22.7	18.1
–	trace	5.6	2.9	–		9.2	6.4	–	1.2	2.3	trace	7.0
17.1	35.2	20.6	7.5	39.5	23.0	30.5	22.0	24.1	28.0	20.7	38.8	26.5
9.9	5.5	6.8	2.1	1.2	3.0	14.5	6.0	9.2	trace	trace	6.4	2.8
10.0	8.9	trace	6.0	20.5	2.8	9.9	0.3	13.4	11.6	9.8	6.9	trace
8.6	20.2	24.1	16.0	9.1	9.1	10.9	24.9	15.2	5.0	5.0	14.0	15.8
3.2	4.1	9.7	11.4	2.2	6.6	1.7	8.4	6.0	4.0	1.8	2.5	4.2
–	trace	1.5	–	–	–	–	–	trace	–	–	–	trace
4.1	2.0	6.1	14.2	1.7	3.6	–	3.1	5.3	2.0	1.8	1.0	3.3
–	–	–	–	–	–	–	–	3.2	trace	–	–	trace
9.6	2.9	5.9	7.8	7.5	3.3	3.4	3.8	4.4	6.5	4.2	1.9	9.9
trace	–	–	–	trace	–	trace	trace	–	trace	trace	–	trace
2.2	4.8	3.1	1.6	1.1	4.4	1.1	2.3	2.0	2.7	1.3	1.5	1.3
2.0	trace	–	–	–	–	–	–	–	5.1	1.8	–	trace
7.4	2.4	trace	4.0	4.3	14.2	1.1	1.6	2.1	4.1	4.4	trace	trace
9.5	1.9	1.6	3.5	4.0	15.1	–	1.0	2.0	5.6	9.7	trace	trace
12.1	2.7	5.6	2.0	6.8	10.3	4.6	7.8	4.2	19.2	30.0	2.5	6.7
0.44	1.10	0.44	0.52	1.57	0.43	1.05	0.52	0.98	0.78	0.58	2.16	0.86

arbon chain length: number of double bonds. The *n*-values represent the methyl end chain from the centre of double bond farthest from the carboxyl end.
/w) of total fatty acids.
total lipid.

TABLE VI

Fatty acid composition of monogalactosyldiglyceride from maturing sperm plasma membrane

The data shown are mean of three experiments that are within ± 15.0% of the mean. Fatty acids of < 1.0% are listed as 'trace'.

Fatty acids [a]	Caput [b]	Corpus [b]	Cauda [b]
14:0	1.9	6.2	9.5
16:0	14.9	13.9	14.0
16:1($n-7$)	3.2	–	–
18:0	3.9	11.9	11.9
18:1($n-9$)	28.0	19.5	16.8
18:2($n-6$)	11.1	11.0	17.8
18:3($n-3$)	trace	–	5.0
20:2	trace	–	–
20:3($n-9$)	trace	–	–
22:0	1.0	0.9	–
20:4($n-6$)	13.1	2.2	2.1
22:2	–	trace	–
20:5($n-3$)	6.0	8.0	8.5
22:5($n-6$)	9.8	9.2	6.0
22:5($n-3$)	2.6	8.8	3.6
22:6($n-3$)	3.7	7.5	4.8
Saturated/ unsaturated	0.28	0.49	0.55

[a,b] See footnote to Table V.

Component PL

Phospholipid constituents of sperm membrane were PC, PE and sphingomyelin (Table VII). PC was the major component and PE was the second major PL.

TABLE VII

Phospholipid composition of maturing sperm plasma membranes

The data shown are mean of three experiments that are within ± 10.0% of the mean.

Phospholipids [a]	Caput	Corpus	Cauda
Total phosphatidyl ethanolamine (PE)	364.0	215.0	199.0
DiacylPE	84.7	41.6	28.4
AlkylacylPE	84.7	48.5	56.0
AlkenylacylPE	195.0	125.0	112.0
Total phosphatidyl-choline (PC)	373.0	381.2	284.0
DiacylPC	50.8	125.0	28.4
AlkylacylPC	84.7	55.0	57.0
AlkenylacylPC	237.2	200.0	200.0
Sphingomyelin	110.1	98.0	85.2

[a] Expressed as μg/mg protein.

The content of PE, PC and sphingomyelin in membrane decreased by approx. 45%, 25% and 20%, respectively, during the epididymal transit of sperm. Three categories of PC and PE were identified. They were the common diacyl form along with the alkylacyl and alkenylacyl forms, the last species being the major constituent in both PC and PE at all stages of sperm maturation. Of all the membrane phospholipid species, diacyl PE decreased most strikingly (approx. 65%) during the epididymal maturity of sperm.

TABLE VIII

Fatty acid compositions of total PE, total PC and Sph of sperm plasma membrane phospholipids

The data shown are mean of three experiments that are within ± 15.0% of the mean for most of the components. Fatty acids of < 1.0% are listed as 'trace'.

Fatty acids [a]	Caput [b]			Corpus [b]			Cauda [b]		
	PE [c]	PC [c]	Sph [c]	PE	PC	Sph	PE	PC	Sph
14:0	18.0	5.9	30.4	20.0	3.0	35.1	23.8	12.0	37.3
16:0	24.6	31.2	33.4	34.0	36.1	34.7	36.7	30.5	36.2
16:1	trace	–	trace	–	–	trace	trace	trace	trace
18:0	10.3	10.0	9.8	6.5	14.2	10.7	11.0	12.6	8.2
18:1($n-9$)	15.3	20.8	13.0	9.0	8.5	9.2	5.2	14.1	12.3
18:2($n-6$)	12.3	13.2	trace	7.3	8.4	2.0	10.6	10.0	1.0
18:3($n-3$)	3.1	2.0	2.6	3.3	4.4	2.5	4.1	3.4	trace
20:2	trace	–	trace	trace	–	trace	–	trace	1.2
20:3($n-9$)	–	trace	–	–	trace	–	–	–	–
22:0	trace	–	–	trace	trace	trace	trace	trace	trace
20:4($n-6$)	6.7	5.2	1.5	6.7	8.0	1.5	trace	6.6	–
22:2	–	–	–	trace	trace	–	–	–	–
20:5($n-3$)	1.3	–	1.2	2.7	3.4	1.0	trace	3.3	trace
21:5($n-3$)	trace	trace	–	1.0	–	–	trace	trace	trace
22:5($n-6$)	1.0	1.4	3.4	1.8	2.0	1.0	1.2	trace	1.2
22:5($n-3$)	1.3	1.6	3.0	1.5	2.5	1.0	1.6	1.4	trace
22:6($n-3$)	5.1	7.4	trace	5.2	8.5	trace	4.0	4.4	trace
Saturated/ unsaturated	1.13	0.89	2.78	1.57	1.17	4.15	2.52	1.25	4.52

[a,b] See footnote to Table V.

[c] PE, phosphatidylethanolamine; PC, phosphatidylcholine; Sph, sphingomyelin.

Fatty acid composition of PL

Fatty acid composition of total PC and PE and sphingomyelin are shown in Table VIII. The fatty acid profile of all the membrane bound PL, was markedly altered during sperm maturation. The most characteristic feature was the predominance of the saturated fatty acids in the PL of the mature sperm membrane. Of all the PL, spingomyelin showed maximal level (approx. 80%) of the saturated fatty acids. The major saturated fatty acid of the PL, were C_{14} and C_{18} fatty acids. The ratio of saturates to unsaturates increased greatly during sperm maturation. The major unsaturated fatty acids of PL were oleic ($18:1(n-9)$), linoleic ($18:2(n-6)$) and arachidonic ($20:4(n-6)$) acids.

Discussion

Extensive studies have been carried out for the last decade to elucidate the biochemical basis of the regulation of flagellar motility using goat epididymal sperm model (for review see Ref. 3). Methodologies have been developed for measuring the intactness of isolated sperm [60] and iodination of viable maturing sperm [26], and highly purified plasma membranes from the mature and immature spermatozoa [25,27]. Anti-sticking factor (ASF) and forward motility-stimulating factor (FMSF) have been identified using this cell model [61,62]. Some of our recent studies on the maturing goat sperm center around the functional characteristics of the sperm plasma membrane with special reference to cell adhesion [61], cell-surface antigens [6], protein phosphorylation/dephosphorylation mechanisms [3,11,12,63] and lipid-phase fluidity [64]. We have, therefore, used in the present studies a goat sperm model for an indepth analysis of the lipid composition of highly purified plasma membranes isolated from the maturing sperm to establish the possible functions of membrane lipids in the epididymal sperm maturation.

The present study reports extensive analysis of the lipid composition of maturing goat sperm plasma membrane. Phospholipid (approx. 75% w/w) neutral lipids (approx. 15% w/w) and glycolipid (approx. 10% w/w) represented the major sperm membrane lipids. The concentration of these lipids and their fatty acid composition underwent marked alteration during the epididymal sperm maturation. This investigation demonstrates for the first time maturation-dependent alterations of multiple neutral lipids including the novel wax ester and Alk-DAG in the sperm plasma membrane.

Data on the lipid composition of membrane derived from boar [24], ram [23] and goat showed some similarities and dissimilarities in respect of their alteration during sperm maturation. Although PC and PE were the major phospholipids in the sperm membrane of all the species, marked differences were noted in their maturation associated changes. While the PC content of goat sperm membrane decreases (Table VII), it increases in the maturing membrane of ram and boar sperm [23,24]. Sperm membrane PE has been shown to decrease in all the species. Unlike goat (Table VII), the spingomyelin content of the ram and boar sperm membrane has been found to increase during the maturation process. Like the goat sperm membrane, the boar sperm membrane as well possesses diacyl, alkylacyl and alkenylacyl forms of PC and PE (Table VII). The mature goat sperm membrane showed markedly higher sterol content than that of the immature cells (Table II). In this respect, the ram and boar sperm showed striking differences, since the sterol content decreased during maturation in the membranes of these cells [23,24]. The cholesterol/phospholipid ratio increased during the epididymal maturation of ram [23] and goat sperm (Table III), but it decreased in boar sperm [24]. Although the nature of the glycolipids were different in goat and boar [24] sperm membrane, both of them underwent marked decrease during the maturation of the male gametes. The overall fatty acid profile of membrane of maturing goat-sperm was similar to those of boar [24] and ram [23]. At present, little is known regarding the differential maturation-associated changes of the sperm membrane lipids in the different species. Species specificity may be one of the parameters that may be involved in this phenomenon. It may be noteworthy that the earlier workers [23,24] have expressed their data of membrane lipids on the basis of cells numbers, rather than protein content of the isolated plasma membrane. Yields of membrane are likely to differ in different lots of the same cell population as well as in cells of varying physiological states [27]. Consequently, the data of membrane lipid composition of maturing sperm based on cell number [23,24] may not be quantitative. In this report, the results have been expressed on the basis of the protein contents of the isolated plasma membrane, thereby eliminating the technical problem specified above. It thus appears that some of the above-mentioned variation in the lipid composition of the membrane of maturing sperm of different species, may be due to an artifact of expressing of data.

It is well documented that the membrane-bound phospholipids, e.g., PC and PI play an important role in transmembrane signalling of external stimuli exerted by hormones, neurotransmitters etc. [65–67]. Protein kinase C is activated by DAG, a second messenger of the signalling system and the activated kinase propagates the signal to the cellular compartments by phosphorylation of specific proteins [66]. Boar sperm membrane has been shown to possess PI and DAG [24]. Although these lipid components were not detected in the goat sperm membrane, it is possible that these regulators of signal transduction, may as well be present in trace amounts in the goat sperm membrane. 1-*O*-Alkyl-2-acyl glycerols are produced in MDCK cells by the phospho-

lipase-C-mediated hydrolysis of PC [68]. Synthetic alkylacyl glycerols have been shown to inhibit growth of HL-60 promyelocytic leukemia cells and stimulate their differentiation to macrophage-like cells [69]. The alkylacyl glycerol inhibits DAG-dependent protein kinase C activity [70]. This inhibitory action of the 1-*O*-alkyl-2-acyl glycerol on the protein kinase C may form the basis of its biological activity [70]. Although the ether lipid Alk-DAG is known to be present in the mammalian cells [71], only recently its presence has been demonstrated in the plasma membrane of a cell type (goat spermatozoon) (unpublished results). Biological significance of the ether lipid is largely unknown. Lipase may act on the membrane bound Alk-DAG to cause the formation of alkylacyl glycerol, which may inhibit protein kinase C. It is possible that the Alk-DAG, because of its structural similarity with the alkylacyl glycerol, may directly modulate the activity of protein kinase C. It thus appears that the membrane bound Alk-DAG or its metabolites may play an important role in signal transduction in the mammalian cells.

It is of interest to note that the PE/PC ratio of sperm membrane decreases significantly (approx. 30%) during sperm maturation (Table VII). A high content of PE destabilises membrane bilayer, because of its preference for hexagonal configuration [72]. Thus, relatively low level of PE compared to PC would be expected to favour the more stable bilayer configuration in mature sperm membrane. More than 75% of PE and PC of goat sperm membrane contained ether linkages (Table VII). The ether linkages are known to be stable to the hydrolytic actions of lipases and because of the steric hindrance they retard the rate of hydrolysis of acyl moieties at the *sn*-2 position of the same molecules by the lipases [73]. Selivonchick et al. [74] have shown that ether lipid provides an important metabolically stable membrane constituent during sperm maturation. The degradation of 1-*O*-alkyl- and 1-*O*-alkenyl species of PL by phospholipase C leads to the formation of alkoxyacyl glycerols [62,75] that may function as negative modulators of protein kinase C [67,70]. Like the Alk-DAG, as discussed in a previous section, the 1-*O*-alkyl-2-acyl and 1-*O*-alk-1′-enyl species of PL may also play some role in transmembrane signalling.

The lipid constituents of membrane interact with each other to determine the structural and functional characteristics of the biomembranes. It is well documented that cholesterol and unsaturated fatty acids have great condensing effect on biomembranes and phospholipids reduce the condensing potential of these hydrophobic constituents [14]. Sphingomyelin is known to exert a rigidifying effect on biomembranes. It has also been shown to interact preferentially with cholesterol [76–78], which may possibly amplify the ordering effect [79,80]. Docosahexaenoic acid (22 : 6(n − 3)) is an important polyunsaturated fatty acid of boar [24], human and goat sperm membrane and it has been implicated to regulate free fatty acid utilisation during the epididymal maturity of sperm [81]. The six double bonds of docosahexaenoic acid affect the conformation of the PL in the lipid bilayer by limiting the motional freedom and the condensing potential of cholesterol [14]. MGDG identified in the goat sperm membrane has also been demonstrated in sarcoplasmic reticulum membrane, where it forms hexagonal (II) phase and stimulates Ca^{2+} pump activities [82]. It thus appears that the observed alterations of the above-mentioned lipid constituents of goat sperm membrane during the epididymal transit (Tables II, III and VII) will greatly influence the structure and function of the sperm membrane. Little is known about the biochemical basis of the alteration of the sperm membrane lipids as a consequence of maturation. The modulation of membrane lipids may be caused by uptake of lipids from the intracellular pool and/or intramembranous alteration mediated by various membrane associated lipases. Lipid exchange proteins have been demonstrated in membrane of mammalian cells [83]. It is possible that the goat sperm membrane may as well contain lipid exchange proteins to permit uptake of lipid constituents from the exogenous fluids, such as epididymal plasma.

Recent studies from our laboratory have shown that the lipid-phase fluidity of the sperm plasma membrane decreases significantly during the epididymal maturity of the male gametes [64]. It is well documented that the membrane microviscosity is greatly influenced by cholesterol/phospholipid and saturated/unsaturated fatty acids ratios of the lipids [14]. It thus appears that the observed marked increases in the above-mentioned ratios of lipids and fatty acids during the epididymal sperm maturity (Tables III, V, VI and VIII) is primarily responsible for the maturation-dependent changes in the sperm membrane fluidity [64]. The maturation-dependent alteration of the lipid composition of membrane derived from goat, as well as ram [23] and boar [24], may thus bring about membrane structure alteration to permit expression of motility and fertility characteristics in the male gametes. The altered fluid state of the membrane consequent upon maturation may have an important role in the regulation of membrane function with special reference to ion transport, activity of membrane-bound enzymes, masking and unmasking of cell surface macromolecules, sperm capacitation, acrosomal reaction and cell fusion during fertilisation.

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