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Human prostasome membranes exhibit very high cholesterol/phospholipid ratios yielding high molecular ordering

Gösta Arvidson [1], Gunnar Ronquist [2], Göran Wikander [3] and Anna-Carin Öjteg [1]

[1] *Department of Medical and Physiological Chemistry, Biomedical Center,* [2] *Department of Clinical Chemistry, University of Uppsala, Uppsala, and* [3] *Department of Physical Chemistry, University of Umea, Umea (Sweden)*

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Lipid analysis and ESR studies were carried out on prostasomes isolated from human semen. Cholesterol plus phospholipids amounted to approximately 0.80 μmol per mg protein with a striking quantitative domination of cholesterol over the phospholipids, the molar ratios of cholesterol/sphingomyelin/glycerophospholipids being 4:1:1. Saturated and monounsaturated fatty acids were dominating both in the glycerophospholipids and in sphingomyelin. The order parameters, *S*, deduced from ESR spectra of spin-labelled fatty acids incorporated into prostasome membranes were very high, viz. 0.75 for 5-doxylstearic acid and 0.30 for 16-doxylstearic acid at 25 °C. Slightly lower values were obtained for the spin-labelled fatty acids when they were incorporated into dispersions of extracted prostasome lipids or into synthetic lipid mixtures of similar composition. The highly ordered lipids in the prostasome membrane thus seemed to be minimally perturbed by proteins in the membrane and ESR spectra showed no signs of immobilized lipids.

Introduction

Seminal plasma represents the fluid portion of semen in which spermatozoa are bathed at the time of ejaculation. It provides a medium for the motility of spermatozoa, and several of its constituents are known to assist the spermatozoa in fertilizing the egg. Seminal plasma is a mixed fluid consisting of contributions from different secreting glands. The prostatic contribution to an average ejaculate (3.5 ml) usually is 0.5–1.0 ml [1]. The prostatic fluid not only contains soluble constituents but also small corpuscular structures or organelles [2] later denoted prostasomes [3]. Hence, prostasomes are an integral part of the seminal plasma composition and they can be isolated upon preparative ultracentrifugation and recovered in a distinct band at gradient centrifugation [3]. The physiological role of these organelles is not elucidated but the membrane encasing them contains several enzyme systems [4]. The prostasomes may participate in the liquefaction process [5] and may be important for sperm forward motility [6,7]. The protein composition of the membrane surrounding the organelles seems to be complex [3], and analysis with two-dimensional gel electrophoresis revealed a composite protein pattern with most of the proteins in the molecular mass range of 10–90 kDa [8]. In the present report we have investigated the lipid composition and structure of prostasome membranes.

Materials and Methods

Preparation of prostasomes from human semen samples. Semen was obtained from men who were referred to the fertility clinic for investigation of fertility. After semen liquefaction (30–45 min at room temperature), sperm and possible cell debris were separated from seminal plasma by centrifugation for 20 min at 1000 × *g*. The seminal plasma was subsequently ultracentrifuged for 2 h at 105 000 × *g*. The pelleted material thus obtained (pellet II) was resuspended in 500 μl of 30 mM Tris-HCl buffer containing 130 mM NaCl (pH 7.6). This pellet II suspension (0.5 ml–2.0 ml, sometimes several suspensions were pooled together) was further purified on Sephadex G-200 in accordance with a previous investigation [9] to separate an amorphous protein substance [3] from prostasomes. The eluted prostasomes on Sephadex G-200 [9] were ultracentrifuged for 2 h at 105 000 × *g* and the pelleted material containing 'pure'

Correspondence: G. Ronquist, Department of Clinical Chemistry, University Hospital, S-751 85 Uppsala, Sweden.

prostasomes was resuspended in 600 μl of isotonic buffer either consisting of Krebs-Ringer bicarbonate (KRB) buffer or the aforementioned isotonic Tris buffer. Protein was determined in triplicate according to Lowry et al. [10] with bovine serum albumin as standard. The triplicate determinations agreed within ±2%.

Ultrasonic treatment of prostasomes. A sample dispersion (0.6 ml) consisting of prostasomes in KRB buffer was placed in a glass test tube in ice and ultrasonic treatment was done for 10 and 40 min, respectively, at 40 kHz and an input power of 100 W (Sonifier II Ultrasonic Cell Disruptor, Branson Ultrasonics S.A., Switzerland). The ice-bath was exchanged at least once in order to keep the temperature about 4°C. The prostasome suspension was again ultracentrifuged for 2 h at $105\,000 \times g$ after this treatment and the pellet was resuspended as given above for enzymatic and lipid analyses.

Enzyme assays. The Zn^{2+}-dependent ATPase activity in prostasomes was determined in a buffered medium at pH 6.0 maintaining an ATP/Zn^{2+} ratio of 2:1 in accordance with a previously described method [4]. The enzyme activity was expressed in μmol orthophosphate released per mg protein and 20 min at 37°C. The aminopeptidase assay was according to the method of Laurell et al. [11] using *N*-succinyl-L-alanyl-L-alanyl-L-alanine-*para*-nitroanilide $(Suc(Ala)_3pNA)$ as substrate. The aminopeptidase activity was expressed in units per mg protein. One unit corresponded to the activity of I U elastase (EC 3.4.21.11, type I, No E-1250, Sigma Chemical Co., U.S.A.) per min at 37°C.

Lipid analysis. The prostasome lipids were extracted with chloroform/methanol, (1:1, v/v). One volume of the prostasome suspension (KRB buffer) was mixed with 6 vols. of chloroform/methanol. Then 2 vols. of 0.15 M NaCl acidified to pH 4 were added. Two phases were obtained. The lower, chloroform phase was collected and the upper phase was washed once with chloroform which was then combined with the original chloroform phase. Thin-layer chromatography of the extracted lipids was carried out on plates precoated with 0.2 mm thick layers of silica gel (Merck AG, F.R.G.). Before use the adsorbent layers were purified by running the plates in a mixture of chloroform/methanol/acetic acid/water (25:15:4:2, v/v). The plates were then activated for 2 h at 120°C. Samples from prostasome preparation I (see Table I) were analyzed by one-dimensional chromatography with chloroform/methanol/acetic acid/water (20:20:4:3, v/v) as the mobilic phase. The other samples were analyzed by two-dimensional chromatography on silica gel containing magnesium acetate [12]. Impregnation of the precoated plates was done by spraying the plates with a 1.2% (w/v) solution of magnesium acetate. The plates were then activated at 120°C for 3 h. Developing solvent in the first direction was chloroform/methanol/25% ammonia/water, (65:35:5:2, v/v). After drying *in vacuo* for 30 min the plates were developed in the second direction with chloroform/methanol/acetic acid/water (60:30:8:3, v/v). The phospholipids were visualized with dichlorofluorescein and isolated for phosphorous determination as previously described [13]. The lipids were identified by comparing their chromatographic mobilities with those of authentic standards supplied by Sigma Chemical Co., U.S.A. and Avanti Polar Lipids, U.S.A.

Fatty acid methyl esters for gas-liquid chromatography were prepared by treating total prostasome lipids with 0.1 M NaOH in methanol for 15 min at room temperature. Methyl esters originating from the glycerolipids and unreacted lipids, mainly sphingomyelin, were separated by thin-layer chromatography and isolated. The sphingomyelin fraction was transesterified at 65°C for 4 h in methanol containing 2.5% (v/v) sulphuric acid and the resultant methyl esters were purified by thin-layer chromatography. BHA (2,[3]-*tert*-butyl-4-hydroxyanisol) at a concentration of 50 mg/l was added as an antioxidant to the methanol used in both methylating reactions.

The fatty acid methyl esters were analyzed by gas-liquid chromatography on a bonded FFAP fused silica glass capillary column (Quadrex Corporation, U.S.A.). Shimadzu GC-8A gas chromatograph equipped with a flame ionisation detector and an electronic integrator was used. Gas-liquid chromatography reference standards obtained from Nu Chek Prep, Inc., U.S.A. were used both for identifying the fatty acid methyl esters and for calibrating the response of the detector-integrator unit.

Cholesterol was determined enzymatically with the Monotest™ kit (Cat No. 237 574) supplied by Boehringer Mannheim GmbH, F.R.G. Phospholipids were quantified by phosphorous determinations according to Chen et al. [14]. The reported cholesterol and phospholipid values were the means of duplicate determinations which agreed closely (±3%).

ESR measurements. 5-Doxylstearic acid and 16-doxylstearic acid were purchased from Molecular Probes Inc., U.S.A. Incorporation of doxylstearic acid into prostasomes was achieved by vigorously shaking a prostasome suspension in a glass vial where doxylstearic acid had been deposited as a dry film. The concentration of doxylstearic acid was chosen to yield a (prostasome phospholipid)/(doxylstearic acid) molar ratio of 100. Doxylstearic acid was also added at the same concentration to extracted prostasome lipids or to synthetic lipid mixtures. The lipids were mixed with doxylstearic acid in chloroform/methanol solution and then taken to dryness under a stream of N_2 and dispersed in 50 μl distilled water. Each prostasome and lipid sample was transferred to two glass capillary tubes which were sealed and placed together in the ESR

spectrometer. All ESR spectra were registered at 25 and 37°C with a Varian model E-109 X-band (9 GHz) spectrometer by using an E-238 (TM_{110} mode) cavity. Temperature regulation was achieved by means of a model V-6040 variable temperature controller. The modulation frequency was 100 kHz and the modulation amplitude was always kept less than 0.5-times the linewidth of the central peak in each spectrum. The spectrometer was interfaced to a Zenith-111-32-PC as outlined by Lipscomb and Salo [15].

Calculation of order parameters. For anisotropic and sufficiently fast motion of the spin label on the ESR time-scale, the variation in spectral anisotropy is quantitatively best described in terms of an order parameter, S. This quantity is related to the time-averaged amplitude of angular motion and is given by the following expression:

$$S = \{3\langle\cos^2\theta\rangle - 1\}/2$$

θ is the instantaneous angular orientation of the chain segment relative to the normal of the aggregate surface. The brackets, $\langle\rangle$, imply time-averages of the angle (for references see Ref. 16).

Experimentally the order parameter is given by the ratio of the spectral anisotropy in the aggregate ($A_{\parallel} - A_{\perp}$) to the maximum obtained in a rigidly oriented system (defined by A_{xx}, A_{yy}, A_{zz}, the principal values of the spin label hyperfine tensor). The order parameter, S, can be calculated directly from the spectrum by means of the following expression [17]:

$$S = \frac{A_{\parallel} - A_{\perp}}{A_{zz} - 0.5(A_{xx} + A_{yy})} \cdot \frac{a'_0}{a_0}$$

where $A_{\parallel}$ and $A_{\perp}$ are the outer and inner extremes of the recorded spectrum (cf. Fig. 1). The polarity correction term a'_0/a_0, is introduced due to the hyperfine splitting dependence of the polarity of the label environment, where $a_0 = (A_{\parallel} + 2A_{\perp})/3$ and $a'_0 = (A_{xx} + A_{yy} + A_{zz})/3$

The value $A'_{\perp}$, as determined from the spectrum, has to be corrected to give the true $A_{\perp}$. The correction is given by:

$$A_{\perp} = A'_{\perp} + 1.4\{1 - (A_{\parallel} - A'_{\perp})/[A_{zz} - 0.5(A_{xx} + A_{yy})]\}$$

for $S > 0.45$ [18]

and by

$$A_{\perp} = A'_{\perp} + 0.8 \quad \text{for } S < 0.45 \text{ [17]}$$

Correction of $A_{\perp}$ has been performed according to the above equations when calculating order parameters for the actual systems. The error in the calculated order parameters is approximately 0.01 for $S > 0.3$ according to the standard deviation of repeated measurements on different samples from the same preparation. For $S < 0.3$ the error is estimated to be 0.03.

Results

Major biochemical features of prostasomes are presented in Table I. Lipid composition patterns of these organelles were not qualitatively different from those subjected to ultrasonication for different times. Nor was there any substantial decrease in ATPase and aminopeptidase activities by this treatment (Table I). Measured quantities of phospholipid and cholesterol relative to protein content were rather constant in the four different preparations (Table I). The phospholipid-to-protein ratio was about 0.27 (μmol/mg) and the cholesterol-to-phospholipid molar ratio was high and amounted to about 2.0 (Table I). Phospholipid compositions of prostasome preparations are presented in Table II. Sphingomyelin was the predominant phospholipid class in all preparations, representing nearly half of total phospholipid measured. Phosphatidylethanolamine and phosphatidylserine were relatively high and

TABLE I

Enzyme activities and lipid content of prostasomes

The prostasome lipids were extracted and analyzed for phosphorus and cholesterol as described in Methods. Prostasomes for four different preparations, designated I, II, III and IV were analyzed. Ia and Ib signify prostasomes from preparation I that had been sonicated for 10 and 40 min, respectively.

Sample	ATPase (μmol·mg^{-1}·20 min^{-1})	Aminopeptidase (units·mg^{-1}·min^{-1})	Phospholipid (μmol P/mg protein)	Cholesterol (μmol/mg protein)	Cholesterol / Phospholipid (mol/mol)
I	9.61	3.88	0.24	0.46	1.92
Ia	9.03	3.67	0.29	0.55	1.90
Ib	8.26	2.77	0.29	0.54	1.88
II			0.29	0.67	2.31
III			0.23	0.46	2.00
IV			0.25	0.54	2.16

TABLE II

Phospholipid composition of prostasomes

The lipids were separated by one-dimensional (samples I, Ia and Ib) or two-dimensional (samples II, III) thin-layer chromatography and the phosphorus content of the fractions was determined. Possibly occurring plasmalogens are included in the corresponding phospholipid classes in the table. Samples are designated as in Table I.

Phospholipid class	Sample				
	I	Ia	Ib	II	III
	% of recovered P				
Phosphatidylethanolamine	16.2	17.3	15.5	20.0	16.0
Lysophosphatidylethanolamine	7.9	9.7	9.9	6.8	8.1
Phosphatidylinositol	15.6	16.7	14.7	3.2	2.7
Phosphatidylserine				14.0	11.0
Lysophosphatidylserine	9.8	10.2	9.0	0.7	1.1
Phosphatidylcholine				8.3	7.9
Lysophosphatidylcholine	4.0	4.1	4.1	4.1	3.3
Phosphatidic acid	1.8 [a]	1.1 [a]	1.6 [a]	0	0.8
Sphingomyelin	44.7	40.9	45.2	43.7	48.5
	% of applied P				
Recovery	99	92	95	107	107

[a] Includes origin of the thin-layer chromatogram.

also lysophospholipids occurred in appreciable concentrations while phosphatidylcholine and especially phosphatidylinositol were found at low levels in all preparations (Table II). The relative percentages of the fatty acids are given in Table III.

In the glycerolipids three fatty acids were quantitatively dominating, viz. palmitic, stearic and oleic acid which together accounted for 80% of the total fatty acids. One third of the fatty acids in sphingomyelin was palmitic acid. Remaining fatty acids consisted largely of C_{18}–C_{24} saturated and monounsaturated fatty acids (Table III).

TABLE III

Fatty acid composition of prostasome lipids

Methyl esters of the fatty acids of total glycerolipids and of sphingomyelin were prepared and analyzed by gas-liquid chromatography as described in the text.

Fatty acid	Composition (% by weight)	
	glycerolipids	sphingomyelin
16:0	21.9	32.0
18:0	23.5	8.2
18:1	33.7	11.6
18:2	3.9	0.9
20:0	1.0	8.4
20:1	3.3	–
20:2	1.1	–
20:4	2.8	2.3
22:0	–	12.3
22:6	3.1	–
24:0	–	10.2
24:1	–	8.7
Unidentified	5.8	5.4

Calculated order parameters, S, of 5-doxylstearic acid and 16-doxylstearic acid in prostasomes, extracted prostasome lipids and synthetic lipid mixtures are given in Table IV. From this table it appears that the order parameters of each one of the two different labels are rather similar for intact prostasomes, extracted prostasome lipids as well as synthetic lipid mixtures containing an appreciable amount of cholesterol. This indicates that the packing of the membrane lipid is disturbed very little by the presence of the membrane protein. It is also obvious that cholesterol in the synthetic lipid mixtures increased the order parameter of the label to values being close to those found for intact prostasomes.

By calculating a_0, the isotropic hyperfine splitting constant for a specific label, it is possible to get an estimate of the polarity of the investigated systems (cf. the right column of Table IV). The a_0 values in Table IV indicate that 5-doxylstearic acid is situated in a region with a rather high polarity. 16-Doxylstearic acid on the other hand probes a region in the centre of the membrane where the polarity is low. For a comparison it may be mentioned that typical values for a_0 are 15.6 in water, 15.2 in ethanol and 13.9 in undecane [19]. From the table it may also be concluded that rather small differences in polarity existed between intact prostasomes and lipid mixtures.

A relatively low lipid/protein ratio as found here should facilitate the detection by ESR of immobilized lipid components directly associated with integral membrane proteins. However, the spectrum of 16-doxylstearic acid in intact prostasomes showed no signs of immobilized components (cf. Fig. 1B, upper curve). It seemed less probable that the low lipid/protein ratio was attributable to any 'extra' protein from the interior

TABLE IV

Order parameters, S, and isotropic hyperfine splitting constants, a_{iso}, of 5-doxylstearic acid (5-NS) and 16-doxylstearic acid (16-NS) added to prostasomes and to dispersions of extracted prostasome lipids or synthetic lipid mixtures

The samples were prepared as described in the text. Lipid mixture I and II consisted of cholesterol/sphingomyelin/dioleoylphosphatidylcholine/dioleoylphosphatidylethanolamine in the molar ratios 4:1:0.4:0.6 and 0:1:0.4:0.6, respectively. The order parameter, S, and the isotropic hyperfine splitting constant, a_{iso}, were calculated as described in the text.

Sample	Order parameter, S		Isotropic hyperfine splitting constant, a_{iso} (G)		Temperature (°C)
	5-NS	16-NS	5-NS	16-NS	
Prostasomes	0.75	0.30	15.4	13.9	25
Prostasome lipids	0.71	0.25	15.3	13.9	25
Lipid mixture I	0.65	0.24	15.3	14.1	25
Lipid mixture II	0.58	0.11	14.9	13.9	25
Prostasomes	0.65	0.28	15.3	14.1	37
Prostasome lipids	0.62	0.23	15.1	14.0	37
Lipid mixture I	0.55	0.22	15.2	14.2	37
Lipid mixture II	0.43	0.09	14.8	14.3	37

of the prostasomes, since no release of proteins was observed after disintegration of the prostasome membrane structure by ultrasonication [3].

Discussion

Cholesterol is known to be particularly abundant in plasma membranes which contain saturated phospholipid acyl chains and have a high content of sphingomyelin (see, for example, Ref. 20). Our results on the lipid composition of prostasomes fit well with this picture except that the very high cholesterol/phospholipid ratio of 2 in prostasomes by far exceeds the ratio of 0.8–1.3 reported for cholesterol-rich membranes including myelin [21]. We have no indication for an extramembranous contribution of cholesterol from the interior of the prostasomes that could account for the very high cholesterol/phospholipid ratio found by us. Hence, the prostasomes appeared ultrastructurally pure [3,7] and the presence of ADP and GDP in prostasomes has been established [22]. These nucleotides may exist within the prostasomes in a macromolecular complex that involves stacked nucleotides held together by divalent cations through the phosphate group giving the

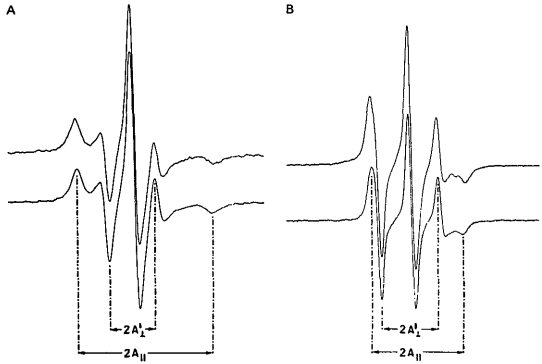

Fig. 1. ESR spectra. In A 5-doxylstearic acid was added to prostasomes (upper curve) and to extracted prostasome lipids (lower curve). B shows corresponding spectra obtained with 16-doxylstearic acid. The spectra were recorded at 37 °C and the scan range is 100 G in all spectra. $A'_\perp$ and A_{II} are the outer and inner extremes used in the calculations of the order parameters, S. (see text and Table IV).

prostasomes their typical electron-dense pattern [22]. Accordingly, there are no ultrastructural evidences of cholesterol or other lipid-containing structures within the prostasomes. Also, the preserved cholesterol/phospholipid ratio after ultrasonication of prostasomes may be contradictory to the occurrence of an additional pool of free cholesterol within the prostasomes. The lipid composition of the plasma membrane of the spermatozoa is very different from that of the prostasome membrane in spite of the fact that spermatozoa and prostasomes share the same external medium. In human spermatozoa [23] the plasma membrane contains substantially less sphingomyelin and more phosphatidylcholine while the representation of phosphatidylethanolamine is about the same as in the prostasome membrane. The cholesterol/phospholipid ratio is 0.83 in the plasma membrane of the human spermatozoon [24].

Since the ordering of lipids in membranes is greatly influenced by the cholesterol and sphingomyelin content and by the degree of saturation of the phospholipid acyl chains [25–29] one would expect the lipids in the prostasome membrane to be highly ordered. This conclusion is confirmed by the spin labelling experiments showing that the order parameters for prostasomes and extracted prostasome lipids are very high. It is noteworthy that the order parameters of 5-doxylstearic acid and 16-doxylstearic acid in intact prostasomes differ very little from those obtained in prostasome lipids (cf. Table IV). This means that the lipids in intact prostasomes are very little perturbed by the membrane proteins. It may also be recalled that cholesterol increases molecular ordering in the bilayer without decreasing the lateral diffusion of the lipids [30]. Furthermore, a liquid-crystalline state is induced when cholesterol is added to pure lipids in the gel state yielding rapid translational motion of the lipid molecules even at temperatures below the transition temperatures of the pure lipids [31]. High transition temperatures for the prostasome membrane lipids are indicated by the present data which show a proportionally high content of saturated acyl chains and of sphingomyelin in the prostasome membrane. It is thus possible that cholesterol is of particular importance for maintaining the prostasome membrane in a liquid-crystalline state. The relatively high level of lysophospholipids in the prostasome membrane is remarkable and may be related to the occurrence of a phospholipase A_2 in the prostasomes [8]. If, or to what extent, this high concentration of lysophospholipids is artifactitious cannot be decided from the present data. It should however be born in mind that lysophosphatidylcholine may form lamellar structures together with compounds of a suitable molecular shape such as cholesterol or fatty acids [32–34].

Electron microscopy revealed that human prostasomes are small spherical corpuscles surrounded by a typical trilaminar membrane [2]. Our results indicate that this membrane is a tight and highly ordered structure. This conclusion is consonant with previous findings that the concentration of not only calcium but also of zinc and magnesium is several times higher in the prostasomes than in the surrounding seminal plasma [35,36]. Also, the resistance of prostasomes against mechanical treatment including ultrasonication and changes in osmolarity in the surrounding fluid including hypoosmosis may be related to the unusual membrane architecture reported here.

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