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Lipid domains in the ram sperm plasma membrane demonstrated by differential scanning calorimetry

(phase separation/phase transition/plasmalogens/alkenyl lipid/ether-linked lipid)

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ABSTRACT **Mammalian sperm plasma membranes, in contrast to those of mammalian somatic cells, exhibit a significant fraction of lipid that does not diffuse laterally in the plane of the membrane. This nondiffusing fraction results from lipid–lipid interactions. Similar nondiffusing fractions are found in mixed-lipid model systems that contain coexistent gel and fluid domains. These results suggest that the sperm plasma membrane may also exhibit lateral phase segregations of lipids and may contain significant amounts of gel-phase lipid. In this paper we use differential scanning calorimetry to show that, in contrast to the plasma membranes of mammalian somatic cells, the plasma membrane from the anterior region of the head of ram sperm exhibits at least two major endothermic transitions, one centered at ≈26°C and one centered at ≈60°C. The heats of these transitions are consistent with gel-to-fluid transitions in model membranes. These transitions are observed both in plasma membrane vesicles and in rehydrated lipid extracts made from these vesicles. These results demonstrate that at physiological temperatures the lipids of the ram sperm plasma membrane are segregated into coexistent fluid and gel domains. Since sperm encounter a wide range of temperatures during their development, these phase transitions may be important in establishing dynamic domains of lipid requisite for epididymal storage and fertilization.**

The plasma membranes of mammalian spermatozoa contrast sharply with those of mammalian somatic cells both in their composition and in their biophysical properties. Sperm plasma membranes have unusually high levels of ether-linked lipids or of highly unsaturated fatty acyl groups such as docosahexaenoyl (22:6) (1–3). After leaving the seminiferous tubules in the testes, sperm have largely shut down lipid synthesis, yet they undergo dramatic posttesticular modifications of their plasma membrane lipid composition both during maturation in the epididymis (1, 4) and during capacitation in the female reproductive tract (2). Lipid composition, particularly cholesterol/phospholipid ratios, may in fact play a mechanistic role in capacitation and the acrosome reaction (5, 6). Unlike lipids in somatic cell plasma membranes, a large fraction of sperm plasma membrane lipid is not free to diffuse (7–9). When bilayers are reconstituted from organic-phase extracts of ram sperm plasma membrane (lipid from the anterior region of the sperm head), a large fraction of the lipid is immobile. Klausner and Wolf (10) have found that lipid is completely free to diffuse in homogeneous fluid-phase model membranes, but have observed immobile fractions in model membranes with coexistent gel and fluid phases. Thus, the immobile lipid fractions observed in mammalian sperm may be due to lipid–lipid interactions resulting in lateral phase segregations.

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Lateral phase separations into domains have been demonstrated for model membranes of mixed lipid composition by a variety of biophysical techniques (for instance, see refs. 11–18). Numerous types of lipid states can exist in the same bilayer. These include coexistent gel/fluid, gel/gel, and fluid/fluid regions. Gel/fluid domains are a consequence of a partially melted overall bilayer. The coexisting gel and fluid domains differ in their proportion of high- and low-melting lipids.

The demonstration of lipid domains in mammalian membranes has been problematic. Mammalian membranes have a multitude of lipid components. Domains confined to a small fraction of the total membrane may not be detectable by techniques such as calorimetry or x-ray diffraction. Spectroscopic studies have provided some evidence for the existence of domains in mammalian plasma membranes. These spectroscopic studies either employed probes that sequester or partition into a subset of membrane domains (10, 19, 20) or employed probes that enter all domains but that have a property (e.g., fluorescence excited-state lifetime; ref. 21) that is distinguishable in the different environments (for reviews, see refs. 22 and 23).

The large, nondiffusing fractions that we have observed on mammalian sperm suggest that sufficient gel-phase domains may be present in the sperm plasma membrane to be detected by differential scanning calorimetry (DSC). In this paper, we report results of such a study. DSC was performed on plasma membrane vesicles from the anterior region of the head of sperm and on rehydrated lipid extracts of these membranes. These measurements demonstrate the occurrence in these membranes of at least two major endothermic transitions, one centered at ≈26°C and one centered at ≈60°C. The heats of these "melts" are similar to those observed for gel-to-fluid transitions in model membranes (15). Of particular relevance is the finding that, at physiological temperature, the ram sperm plasma membrane is between transitions indicative of coexistent membrane domains.

MATERIALS AND METHODS

Sperm. Sperm were collected from sexually mature Corriedale rams by using an artificial vagina maintained at 34°C to prevent temperature shock. Immediately after collection, the sperm were washed by centrifugation (500 × *g* for 10 min) in a modified Hanks' balanced salts solution (without Ca^{2+}, Mg^{2+}, phenol red, or sodium bicarbonate; GIBCO). Sperm were counted on a hemacytometer.

Preparation of Plasma Membrane Vesicles and Lipid Extracts. Sperm plasma membrane vesicles were formed as described (9), by nitrogen cavitation using a modification of the procedure of Parks and Hammerstedt (1). This technique produces vesicles derived from the anterior region of the

Abbreviation: DSC, differential scanning calorimetry.
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sperm head with minimal acrosomal and mitochondrial contamination (1, 9). Lipids were extracted from these vesicles as described (9), by a modification of the technique of Bligh and Dyer (24).

Thin-Layer Chromatography. Lipid analysis by thin-layer chromatography was performed using a modification of the system and procedure of Yavin and Zutra (25). Whatman K6 silica gel plates (10 cm × 10 cm) were activated by heating for 1 hr at 70°C. All chemicals used were reagent grade from Sigma and Baker, unless otherwise noted. Lipid (≈50 μg) in 15–20 μl of $CHCl_3$ was then dotted on the lower left-hand corner of the plate 1 cm from the side and bottom. The plate was then run vertically in $CHCl_3$/methanol/methylamine, 65:30:7.5 (vol/vol). The plate was dried with hot air for 3 min, exposed to fuming HCl for 5 min, and sequentially dried with hot (3 min) and cold (2 min) air. Subsequently, the plate was run horizontally in ether/acetic acid, 95:5, and then dried as before sequentially with hot and cold air. The plate was rerun in the horizontal direction in $CHCl_3$/acetone/methanol/acetic acid/H_2O, 50:20:10:15:5, and blown dry for 10–15 min with cold air. The lipids were visualized by exposure to I_2 vapor. Lipids were identified by comparison with lipid standards. For quantitation, individual spots were scraped from these plates and lipid was extracted from the silica with $CHCl_3$/methanol (2:1). This chromatographic system is designed to provide good resolution of inositol glycerophospholipid and serine glycerophospholipid as well as other lipids and, by virtue of the acid-exposure step, distinguishes the alkenyl lipids from lipids containing only fatty acyl or alkyl chains.

Assays for Organic Phosphate, Hexose, and Cholesterol. Organic phosphate was assayed by ashing according to the method of Ames (26) and phosphate was determined by the method of Lanzetta *et al.* (27). Hexose was assayed by the procedure of Dubois *et al.* (28). Total cholesterol was determined by the method of Courchaine *et al.* (29) and Zlatkis *et al.* (30) as described by Kates (31).

Preparation of Samples for DSC. *Plasma membranes.* Plasma membranes were prepared as described above and 0.1% (wt/vol) NaN_3 was added. Prior to loading into the calorimeter pans, the membranes were pelleted by centrifugation at 100,000 × *g* for 30 min. In some cases, a volume of ethylene glycol equal to the volume of buffer was added to the membrane suspension prior to centrifugation.

Extracted lipid. Extracted lipids in $CHCl_3$/methanol solution were dried under nitrogen on a flat glass plate and then put under vacuum for 2 hr. The lipid was then scraped off the plate with a razor blade and transferred to a calorimeter pan. An excess of deionized water (2:1, water/lipid) was added to the pan to hydrate the lipids, and then the calorimeter pans were hermetically sealed. In some cases, the lipids were hydrated in 1:1 ethylene glycol/water (maintaining a 2:1 water/lipid ratio). In order to assure complete hydration, the pans were repeatedly cycled up and down in temperature between 0°C and 80°C. Reproducibility of thermograms was taken as an indication of full hydration of the lipids.

Differential Scanning Calorimetry. DSC was performed as described (32) with a DuPont 1090 thermal analyzer. Average sample mass was 3–5 mg for both membranes and lipid extracts. Scans were performed at 10 C°/min or slower. There was no significant variation in thermograms with scan rate in this range. All samples were scanned at least twice and the thermograms were compared for any differences. Integration of thermograms, determination of onset and completion temperatures, and curve decompositions were carried out using the DuPont General Analysis Utility Program (version 1.0). In the case of thermograms B and C in Fig. 1, baselines of constant slope were subtracted to correct for sample imbalance.

RESULTS

Analysis of Lipid Composition. The lipid composition of plasma membrane vesicles from the anterior region of the ram sperm head is given in Table 1. The major phospholipid is choline glycerophospholipid (57% of total phosphate), 61% of which is the plasmalogen. The second major lipid is ethanolamine glycerophospholipid (9% of total phosphate), 80% of which is the plasmalogen. Several minor components were also identified: phosphatidylinositol and -serine, phosphatidic acid, sphingomyelin, cardiolipin, and neutral lipids. We did not detect measurable amounts of lysophosphatidylcholine, lysophosphatidylethanolamine, or cholesterol sulfate.

The mol % of cholesterol was determined to be 43 ± 3 (4 preparations, 10 samples).

The molar ratio of hexose to phosphate was determined to be 0.65 ± 0.03 (3 preparations, 10 samples).

DSC. In Fig. 1, curve A is the thermogram of an aqueous suspension of lipids extracted from vesicles derived from the anterior region of the head plasma membrane of ram sperm. Two distinct endotherms are apparent. The lower temperature endotherm begins below 12°C and is complete at about 39°C. The higher temperature endotherm begins at about 48°C and is complete at about 74°C.

Thermogram A is that of a sample run in deionized water. Due to the nature of our instrument, a run is not in equilibrium until some 8°C after its commencement. Thermogram A was initiated at the melting point of water, and therefore data are shown beginning from ≈8°C. The onset temperature for the lower melting transition appears to begin below 0°C. In an attempt to determine the onset temperature, calorimetry was performed on extracted sperm lipids hydrated in 1:1 ethylene glycol/water (thermogram B). While hydrating the lipids in ethylene glycol/water has the effect of partially splitting both transitions, possibly because of interaction with glycolipids, it is apparent that the first transition begins at around −8 to −10°C. If we assume that the first transition begins at −8 to −10°C, we can estimate the heats of the first and second transitions in thermogram A as 25 J/g and 9 J/g, respectively.

Curve C is a thermogram of an aqueous suspension of vesicles derived from the anterior head-region plasma membrane of ejaculated ram sperm after thermal denaturation of protein. The results are similar to those obtained with lipid extracts. A lower temperature endotherm is seen extending to about 40°C. A second and a third higher temperature endotherms are seen extending from approximately 60°C to 75°C and 78°C to 88°C, respectively. Curve D shows a

Table 1. Phospholipid composition of the anterior region of the head plasma membrane of ejaculated ram spermatozoa

Lipid	% of total phosphate	% alkenyl
Choline glycerophospholipid	58 ± 4	61 ± 2
Ethanolamine glycerophospholipid	9 ± 2	78 ± 1
Unidentified I*	7 ± 2	ND
Neutral lipid	6 ± 1	ND
Inositol glycerophospholipid	5 ± 2	ND
Unidentified II*	5 ± 1	ND
Serine glycerophospholipid	3 ± 2	ND
Cardiolipin	3 ± 2	ND
Sphingomyelin	2 ± 1	ND
Phosphatidic acid	1 ± 1	—
Unidentified III*†	1 ± 1	ND

Lipids were identified by comparison with known standards. Values are from four determinations and are given as mean ± standard error of the mean. ND, not detected.

*Not lysophosphatidylcholine, lysophosphatidylethanolamine, or cholesterol sulfate.

†Possibly bis(lysophosphatidic acid).

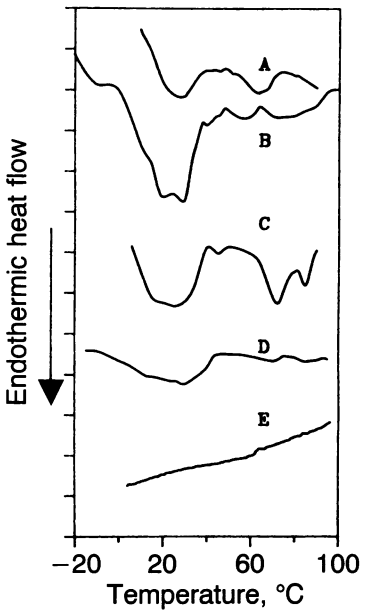

FIG. 1. DSC thermograms of ram sperm plasma membrane: A, rehydrated lipid extract of a plasma membrane fraction from the anterior region of the head; B, similar sample to A rehydrated in 1:1 ethylene glycol/deionized water; C, plasma membrane fraction from the anterior region of the head; D, similar sample to C rehydrated in 1:1 ethylene glycol/deionized water. Thermogram E, human erythrocyte lipids (from ref. 33). Thermograms B and C have had a constant slope subtracted from them to correct for pan imbalance.

thermogram of plasma membrane vesicles run in 1:1 ethylene glycol/water. As in the case of the reconstituted lipid extract, 1:1 ethylene glycol/water disturbs the thermograms. A lower temperature endotherm is observed extending from −4°C to 40°C and two higher temperature endotherms extending from 62°C to 75°C and 78°C to 92°C. For comparison purposes we have included a scan of human erythrocyte lipids in Fig. 1 (thermogram E; data from ref. 33).

DISCUSSION

In this paper, we have shown that the plasma membrane of the anterior region of the ram sperm head differs in its thermal behavior from that reported in mammalian somatic cell plasma membranes, in that it exhibits several prominent endothermic lipid phase transitions.

While transitions due to protein denaturation can be quite dramatic in mammalian somatic cells (34), true lipid transitions are generally weak or nonexistent (34, 35).

The heats of the melts that we have observed compare favorably with gel-to-fluid transitions for pure lipid species [for instance, dimyristoyl, dipalmitoyl, and distearoyl phosphatidylcholines have heats of melt of 33, 49, and 56 J/g, respectively (15)]. While one cannot on the basis of our data alone make definitive statements about the sperm plasma membrane's phase diagram or about the precise nature of the phases present, our data are thermographically similar to those obtained from binary systems such as mixtures of dimyristoyl and distearoyl phosphatidylcholines, which show coexistent gel phases below both melts, coexistent gel and fluid phases between melts, and fluid phase or phases above both transitions (15). At physiological temperatures the sperm membrane is between transitions. This suggests that at physiological temperatures the membrane has coexistent gel and fluid phases. This hypothesis is supported by the observation that these membranes contain large amounts of nondiffusing lipids detected by fluorescence recovery after photobleaching (7–9, 36). Similar nondiffusing fractions have been observed in binary lipid mixtures known to exhibit gel/fluid phase segregations (10).

The question of which features of the sperm lipid composition lead to these transitions and phase segregations remains to be determined. Sperm membranes are not unusual in their cholesterol or glycolipid content. Our lipid composition data for plasma membranes from ejaculated ram sperm are similar to the data of Parks and Hammerstedt (1) for caudal epididymal ram sperm plasma membranes, as well as earlier data on whole sperm lipid composition (37–39), in showing that these membranes contain unusually high amounts of ether-linked lipid compared to somatic cell plasma membranes. Indeed, the major plasma membrane phospholipid species of sperm is 1-alkenyl- or 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine. Parks and Hammerstedt (1) have shown that sperm plasma membranes have very high amounts of polyunsaturated fatty acyl (docosahexaenoyl, 22:6) chains. These polyunsaturated chains are concentrated on the ether-containing choline glycerophospholipids. The other dominant fatty acyl chains are saturated: myristoyl, palmitoyl, and stearoyl. Again, this work is in agreement with studies on whole sperm lipids (2, 4, 37–39). The dominant lipid species in ruminant sperm appears to be a choline-linked glycerophospholipid with a polyunsaturated acyl group (22:6 or 22:5) at the 2 position and a saturated chain such as 16:0 ether-linked as either an alk-1′-enyl or an alkyl chain at the 1 position (3).

Bovine retinal rod outer segment membranes also contain large amounts of 1-palmitoyl, 2-docosahexaenoyl phosphatidylcholine. There is evidence of lateral phase separations in these membranes from fluorescence depolarization studies using parinaric acid (40). While the thermal properties of the L_β to L_α transition are similar for ether- and ester-linked phospholipids, ether lipids exhibit polymorphisms in the gel state, including the formation of interdigitated phases (41, 42). It has also been suggested from ^2H NMR studies that 1-palmitoyl, 2-docosahexaenoyl phosphatidylcholine may form interdigitated phases in the L_α state (43).

Parks and Hammerstedt (1) also reported significant (≈10%) amounts of lyso lipids in their membrane preparations. This was of interest to us since Golan *et al.* (44) reported that lyso lipid concentrations in this range can induce large nondiffusing fractions. However, we have not detected lyso lipids in our preparations.

At this point, it is premature to say precisely what is responsible for the endotherms observed in our studies. One may conjecture however, that at physiological temperatures the large amounts of 1-hexadec(en)yl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine segregate for other lipids to form a fluid phase and that it is the remaining lipid, largely composed of disaturated phospholipids, that melts at a higher temperature.

A further possibility is that glycolipids are playing a major role, in conjunction, perhaps, with other factors already discussed in inducing and controlling phases in the sperm plasma membrane. It has been suggested that glycolipids regulated domain organization in mammalian cells (45). While sperm do not have unusual amounts of glycolipid, glycolipids unique to sperm, such as monogalactosyldiacylglycerol sulfate, have been identified (46–52). The sensitivity of our transitions to ethylene glycol may reflect the importance of glycolipids in these transitions.

A physiological role for the postulated phase segregations is an intriguing consideration. As discussed above, spermatozoa are unique among mammalian cells in that they see a systematic temperature change during their life cycles. The calorimetric data presented here indicate that the ensemble of membrane domains alters as sperm move from the temperature of the testis to that of the epididymis to that of the female reproductive tract. It is only when sperm enter the

female tract and become exposed to 40°C that a significant amount of the plasma membranes are in the fluid state. Sperm are normally stored in the testis and epididymis for considerable lengths of time. Since while in storage they are not actively synthesizing most proteins or lipids, it is advantageous for them to shut down much of their metabolic apparatus (53). Extensive regions of gel bilayer would assist in this storage (16). Thus, the change in temperature when the sperm enter the female may, along with other factors, activate sperm in the female reproductive tract. Resulting changes in membrane lipid domain organization could also play a role in this activation, requisite for fertilization.

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