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Biochimica et Biophysica Acta 1279 (1996) 197–202

BBA Biochimica et Biophysica Acta

Laurdan fluorescence spectroscopy reveals a single liquid-crystalline lipid phase and lack of thermotropic phase transitions in the plasma membrane of living human sperm

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Received 2 May 1995; revised 9 October 1995; accepted 16 October 1995

Abstract

Membrane lipid phase(s), phase coexistence, and thermotropic phase transitions have been investigated in viable human spermatozoa using Laurdan fluorescence spectroscopy. Generalized polarization (GP) values derived from Laurdan excitation and emission spectra confirm that the sperm plasma membrane is a low polar, highly rigid (liquid-ordered) structure, and give evidence that, in the range from 10°C to 42°C, membrane lipids are in a single liquid-crystalline phase. The absence of phase transitions in the same thermal range argues against the hypothesis that the lipid domains previously detected on the sperm surface are produced by lipid lateral phase separation. The above findings are likely accounted for by the high cholesterol to phospholipid molar ratio found in the human sperm membrane. This is the first time that membrane lipid phase and polarity have been detected and quantified in living mammalian spermatozoa.

Keywords: Laurdan; Generalized polarization; Polarization; Fluorescence; Lipid phase; Membrane; (Human spermatozoon)

1. Introduction

In mature sperm, the physical identity of the plasma membrane is dramatically different from that of somatic cells [1]. Although the phospholipid distribution is very similar to that found for example in erythrocyte membranes [2], the presence of up to 50% nondiffusing lipid fractions [3,4] and the high cholesterol content give the sperm membrane a high degree of molecular polarization, mosaicism and rigidity [5]. From intraepididymal transit up to capacitation and acrosome reaction in the female tract, the sperm plasma membrane undergoes relevant maturational events among which are variations in protein and phospholipid composition [6], a reduction in the chain length of fatty acids [7], an increase of fatty acid unsaturation degree [8] and lowering of the cholesterol to phospholipid molar ratio [9]. At further difference with somatic cells, the mammalian spermatozoon experiences a wide thermal gradient during its life cycle; as a consequence, lipid lateral phase separations have been supposed to occur leading to coexistence of fluid and gel lipid domains in the plane of the membrane [1,3]. The above structural changes alter membrane permeability, enzyme activity, fluidity and fusional susceptibility [10]. Various methodologies (fluorescence, resonance and Fourier transform infrared spectroscopy and differential scanning calorimetry) have been applied to investigate the physical state and the thermotropic behavior of membrane lipids in sperm from different mammals. From these studies, dramatic differences have emerged, ranging from three transition temperatures in intact guinea pig sperm [11] and in ram membrane vesicles [12], to two transition temperatures in ram membrane vesicles and lipid extracts [1] and boar membrane vesicles [13], to one transition temperature in boar and bull membrane lipid extracts [14], to a broad and flat lipid phase transition without a definite transition temperature in intact human sperm [11].

The inter- and intraspecies differences found in the thermotropic behavior of sperm surface lipids worth separate considerations. While interspecies differences could be explained by the well known evidence that spermatozoa from different sources exhibit profound compositional differences in terms also of membrane lipids [2,5,14,15], intraspecies differences in the thermotropic behavior of sperm surface lipids could likely be ascribed to both

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SSDI 0005-2736(95)00250-2

different extraction protocols and different measuring techniques. Apart from the data obtained in intact sperm using Fourier transform infrared spectroscopy [11], where the CH_2 signal – unavoidably derived from all sperm lipids – was not selective for the plasma membrane, most investigations on sperm surface lipid phase transitions have been carried out using preparations of membrane vesicles and/or reconstituted lipid extracts. The loss of cytoskeletal connections and substrates (energetic and not) in the first case (membrane vesicles), to which the loss of the native membrane molecular arrangement and of non-lipid membrane components adds in the second case (reconstituted lipid extracts), could have modified the lipid behavior – to such a point that major differences have been found in sperm from the same species [1,12] – and makes uncertain to compare different data from both the physical and biological viewpoint. Therefore, working with intact cells is likely to produce better results than those obtained with membrane and membrane lipid preparations.

Fluorescence spectroscopy offers several advantages for the study of membrane dynamics; among these are the relative simplicity and the high sensitivity. While in model system several probes have well-defined properties in each individual phase, in biological samples the same probes fail in the observation and quantitation of coexisting separate lipid domains of different phase state (the widely used probes DPH and parinaric acids are good examples of this deficiency [16]). Recently, a new probe has been synthesized [17] and characterized [18–20] for studies of membrane structure and dynamics, the 6-dodecanoil-2-dimethylaminonaphtalene (Laurdan). Upon interaction with the membrane the Laurdan molecules localize at the water interface of the bilayer and, at steady-state conditions – similar to what has been reported for TMA-DPH [21] – the lateral and transbilayer partitioning could be considered uniform. In the membrane, the lauroyl portion of the probe interacts with the phospholipid acyl chains, while the naphtalene moiety anchors at the level of the glycerol backbone where it becomes fluorescent [19]. Due to its spectral sensitivity to solvent polarity and dynamics [20], Laurdan shows different excitation and emission spectra in the gel and in the liquid-crystalline phase, so offering an unique way for the lipid phase state as well as phase domain coexistence and interconversion to be detected and quantified [19,22]. An unique advantage of Laurdan spectroscopy is the possibility of using all the steady-state and dynamic information contained in the fluorescence polarization, avoiding the use of polarizers, which appreciably reduce excitation and emission intensities, and with no need of correction for the scattering contribution, generally relevant when using biological materials.

In the present study, the Laurdan spectroscopy has been used to selectively probe the plasma membrane of viable, intact spermatozoa in order to define lipid phase(s) and polarity, and to search for the coexistence of gel and liquid-crystalline phase domains.

Ascertaining structural and dynamic properties of the sperm surface could help shed light on the still largely unknown mechanisms such as antigen exposure, receptor mobility, transmembrane traffic and cell motility, underlying paramount biological gamete functions (i.e., hyperactivation, capacitation, oocyte recognition and acrosome reaction) both in normal and pathological conditions.

2. Materials and methods

2.1. Chemicals

Laurdan was purchased from Molecular Probes (Eugene, OR) and kept at room temperature in the dark as a 2.5 mM stock solution in DMSO; all probe manipulations were carried out in red light. A buffer solution containing (mM) Hepes, 30; $MgSO_4 \cdot 7H_2O$, 1.19; KCl, 5; NaCl, 120; D-glucose, 5.5; pH 7.4, was prepared either without added calcium (washing buffer) or with 1.8 mM Ca^{2+} (working buffer). All other chemicals were purchased from Sigma. The water used in this study was deionized (> 18 kΩ) with a Milli-Q water system (Millipore). Laurdan-labelled liposomes were prepared from dipalmitoylphospatidylcholine (DPPC) and dilauroylphosphatidylcholine (DLPC) (Avanti Polar Lipids, Alabaster, AL) according to [22].

2.2. Spermatozoa

Seminal fluids obtained from healthy, fathered donors with normal semen characteristics [23], were collected into plastic containers and allowed to liquefy at room temperature ($20 \div 22$°C) in the dark. Spermatozoa were isolated by a swim-up procedure [24] and washed twice ($200 \times g$, 2 min each) with working buffer. The final suspension ($(30 \div 40) \cdot 10^6$ cells ml^{-1}) was maintained at room temperature in the dark up to the time of use. Before and after fluorescence readings, sperm viability was assessed by propidium iodide exclusion [25].

2.3. Fluorescence assay and data handling

0.4 μl of Laurdan stock solution was added upon gentle stirring to $20 \cdot 10^6$ spermatozoa suspended in 2 ml working buffer, and cell labeling was allowed to proceed for either 10 min at 39°C or 40 min at 12°C (Laurdan and DMSO final concentrations 0.5 μM and 0.02%, respectively).

Fluorescence readings were carried out in a computer-driven spectrofluorimeter (Kontron SFM25) equipped with a stirring accessory (150 rpm) and thermostated (± 0.1°C) by a circulating water bath. While applying a thermal step gradient (0.7°C min^{-1}), specimen's temperature was continuously measured with a thermocouple inserted into the reading cell. The monochromator band pass was set at 5 nm for both the excitation and emission path. Laurdan

emission spectra were obtained in the range from 420 nm to 530 nm, using both 340 nm and 410 nm excitation wavelengths, while the excitation spectra were obtained in the range from 330 nm to 420 nm, using both 435 nm and 490 nm emission wavelengths. Blank spectra were obtained with unlabeled cells and were subtracted from the spectra of labeled cells. From the spectroscopic data, Laurdan emission and excitation generalized polarization (GP) spectra were derived by calculating the GP value for each emission and excitation wavelength, respectively:

$$\text{emGP} = (I_{410} - I_{340})/(I_{410} + I_{340})$$

where I_{410} and I_{340} are the intensity at each emission wavelength using excitation wavelength of 410 nm and 340 nm, respectively;

$$\text{exGP} = (I_{435} - I_{490})/(I_{435} + I_{490})$$

where I_{435} and I_{490} are the intensity at each excitation wavelength using emission wavelength of 435 nm and 490 nm, respectively [22].

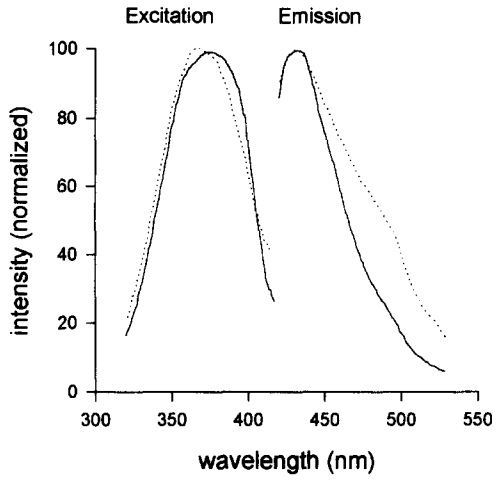

Fig. 2. Normalized Laurdan excitation and emission spectra collected at 10°C (solid line) and 42°C (dotted line). The maximum at 435 nm in the emission spectra and the low intensity of the red band in the excitation spectra are evident. The blue shift in the excitation spectrum and the red band increase in the emission spectrum are expression of accelerated dipolar relaxation processes induced by increased temperature.

3. Results and discussion

The isolation procedure yield a sperm population with > 90% viable and regularly moving sperm. In preliminary experiments (data not shown), $10 \cdot 10^6$ sperm ml^{-1} and 0.5 μM Laurdan – the lack of quantitative data on the lipid content in the entire plasma membrane of human sperm hinders to estimate the actual Laurdan to phospholipid mol ratio – were found to realize the best analytical conditions (independence of GP values from cells to probe concentration ratio and high spectral signal with a low blank signal). Laurdan interaction with spermatozoa did not exert evident cytotoxicity (no increase of propidium iodide nuclear stainability and no decline of sperm motility after probe equilibration).

Laurdan uptake was temperature-dependent, with an apparent steady-state reached within 10 min at 39°C and within 40 min at 10°C (Fig. 1). Normalized Laurdan excitation and emission spectra collected at 10°C and 42°C are reported in Fig. 2. Both the excitation spectra appear as a single, bell-shaped peak. A 10 nm blue shift (from 370 nm to 360 nm) of maximal intensity and a decreased intensity of the red band centered at 390 nm are evident in the excitation spectrum collected at 42°C in respect to that collected at 10°C. In the emission spectra, the maximal intensity is centered at 435 nm at both temperatures, while an evident red band centered at 490 nm appeared only in the emission spectrum collected at 42°C, which results are expression of a temperature-dependent increased frequency of dipole relaxation processes (see below).

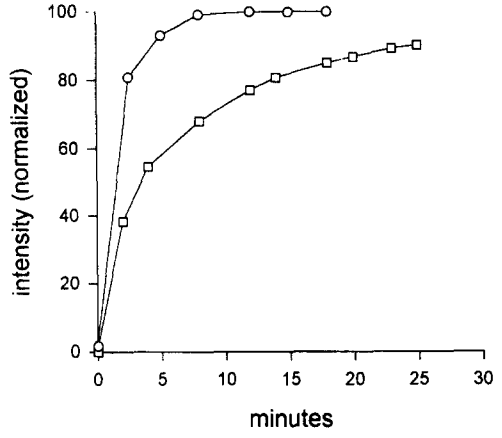

Fig. 1. Time-course of Laurdan (0.5 μM) uptake in human spermatozoa ($107\ ml^{-1}$) at 11°C (□) and 39°C (○).

At 37°C, an $exGP^{340\ nm}$ value of 0.38 ± 0.02 and an $emGP^{435\ nm}$ value of -0.42 ± 0.02 were calculated, indicating a very low rotational freedom degree of water molecules (i.e., high lipid packing) and a low number of hydrogen bonds (i.e., low polarity) in the probe surroundings. The excitation GP spectra values decreased with increasing excitation wavelength, while emission GP spectra values increased with increasing emission wavelength, indicating the presence of a single lipid liquid-crystalline phase. Similar behavioral characteristics were observed for GP spectra collected from 10°C to 42°C, with excitation and emission GP values increasing with decreasing temperature (Fig. 3). In the same figure Laurdan exGP and emGP spectra obtained from liposomes with coexisting gel and liquid-crystalline lipid phases are reported. From the curves it clearly appears that exGP increased and emGP decreased with increasing excitation and emission wavelengths, respectively; such a behavior originates from the photoselection of Laurdan molecules populating gel and liquid-crystalline domains [22].

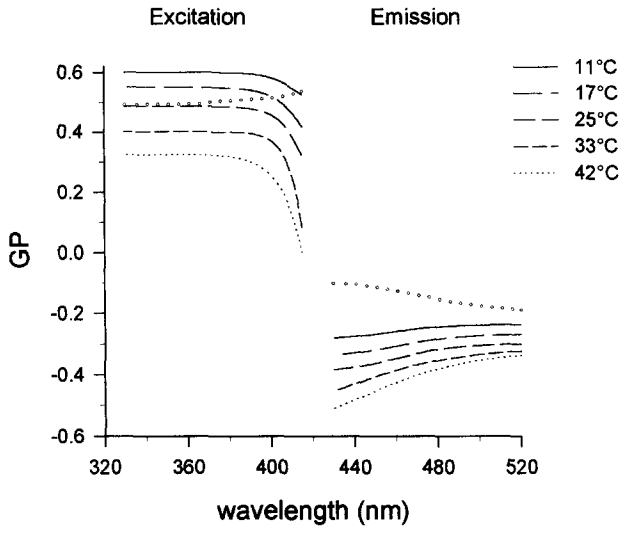

Fig. 3. Laurdan excitation and emission GP spectra obtained at the indicated temperatures. The decrease of exGP with increasing excitation wavelength and the increase of emGP with increasing emission wavelength indicate that a single lipid liquid-crystalline phase is present in the plasma membrane of human spermatozoa. For comparison, Laurdan exGP and emGP spectra obtained at 25°C in the presence of coexistent gel and liquid-crystalline lipid phases in liposomes made of equimolar DLPC-DPPC mixture + 10 mol% cholesterol are shown (open circles).

The linearity of the temperature dependence of exGP values (Fig. 4) indicates that no phase transitions have occurred within the thermal gradient applied.

Recently, the phase state and dynamics of the plasma membrane lipids have been defined and quantified in viable somatic cells using steady-state Laurdan spectroscopy [16,22,26]. By using the additivity rule that holds for polarization, it was supposed that GP values could be resolved into their fractional intensities associated with the gel and liquid-crystalline phases [16]. Further studies from the same authors, however, ascertained that a GP value intermediate between the GP value of the gel and of the

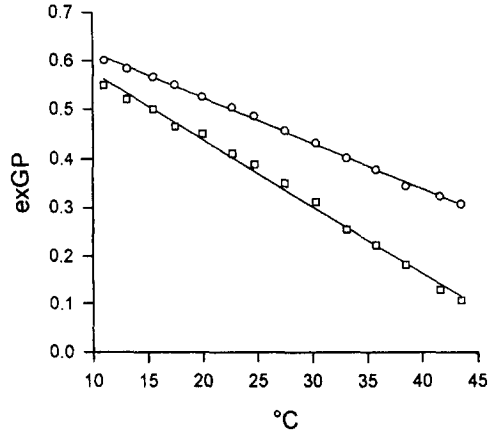

Fig. 4. Temperature-dependence of Laurdan excitation GP values obtained using 340 nm (○) and 410 nm (□) excitation wavelengths. The linearity indicates that no phase transitions have occurred from 10°C to 42°C.

liquid-crystalline phase is not a proof of the coexistence of separate phospholipid phase domains. Instead, GP measurements at various excitation or emission wavelengths can ascertain the coexistence of domains of different phase properties in the membrane plane [27]. In somatic cells, constitutive surface traffic continuously drives labeled membrane portions into the cell interior, resulting in a progressive intracellular probe loading; hence, potentially ambiguous findings could result from Laurdan spectroscopy in living cells. Instead, working with spermatozoa, in which membrane traffic is virtually absent, the above serious drawback does not exist, and the male gamete seems then to offer the best mammalian model in which Laurdan properties can be exploited to study surface lipid dynamics. Laurdan labeling of spermatozoa was temperature dependent and attained an apparent steady-state in a shorter time than that reported for other cell types [22]. This finding was not unexpected, since the absence of membrane traffic in sperm precludes label internalization. In labeled sperm, a maximum at 435 nm appeared in Laurdan emission spectra (Fig. 2), which is typical of the lipid gel phase. The low intensity of the red band seen in the excitation spectra, however, is typical of the lipid liquid-crystalline phase. These spectral characteristics, which would be contradictory in case of a lipid environment only made of phospholipids, are known to be inducible by the presence of cholesterol [16]. Upon increasing temperature, a variation of Laurdan spectra profiles was evidenced. As far as the increase of the red band seen in the emission spectrum is concerned, a temperature-dependent acceleration of kinetics and a higher motional freedom of water molecules – the latter induced by a decreased packing density of the lipid molecules – may account for the increased frequency of dipole relaxation processes which the red band depends on. The blue shift of the excitation spectrum induced by increasing temperature is due to the increased amount of relaxation processes rather than to increased polarity of probe surroundings (the latter should cause in fact a red shift of excitation spectrum) [28]. The shape of the GP spectra and the GP values obtained in this study (Fig. 3), indicate that the lipids of the sperm membrane are in a single liquid-crystalline phase with a high conformational order and low polarity. Again, these findings could likely be accounted for by the known ability of cholesterol to both increase the membrane molecular order and to reduce the bilayer hydration [28]. These data are in accordance with a recent report in which, using EPR methodology, a highly ordered molecular arrangement was detected in the plasma membrane of intact human spermatozoa [29]. The GP spectra obtained at all temperatures studied were comparable in shape, with excitation GP values collected at 410 nm constantly below those collected at 340 nm (Fig. 4), so demonstrating that the lipid liquid-crystalline state is maintained from 10°C to 42°C. The decrease of GP excitation values with increasing temperature reflects an increasing frequency of probe re-

laxation processes, meaning that the membrane interface is being populated by a higher number of faster moving water molecules. The evidence that membrane lipids are constantly in a liquid-crystalline phase argues against the occurrence of thermotropic lipid phase transitions, and rules out the coexistence of gel and liquid-crystalline phase domains in the lipid environment of human sperm plasma membrane, at least in the 10°C to 42°C range. As a matter of fact, the elevated cholesterol to phospholipid molar ratio found in the plasma membrane of human sperm may account for all the above findings. Indeed, very recent data show that at cholesterol concentrations > 15 mol% coexistence of lipid phase domains cannot be detected in preparations made of mixed phospholipids [28]. On a theoretical ground, the relatively high excitation GP values and the relatively low emission GP values are compatible both with a homogeneous lipid liquid-crystalline phase and the coexistence of lipid liquid-crystalline phases with different ordering degree (i.e., lipid domains) [22]. On the basis of previous data, however, the latter hypothesis seems to be the most likely. In particular, by using fluorescence recovery after photobleaching (FRAP), large nondiffusing lipid fractions have been demonstrated in ram [3], mouse [30] and guinea pig spermatozoa [31]. Some of these findings have been interpreted in terms of coexistence of lipid phase domains in the membrane plane. However, since lipid domain area can be as small as $20 \div 50$ Å^2 [22], the much wider area of the laser spot used in FRAP measurements (> 2 μm^2) could cover multiple adjacent domains. As a consequence, the FRAP technique can not discriminate between the coexistence of gel and liquid-crystalline lipid phases and a single phase populated by lipid domains at different fluidity degree.

Lipid domains in the plasma membrane of human spermatozoa have also been identified in freeze-fracture studies. In these studies, cholesterol was visualized by the sterol probe filipin and found to be densely packed in the anterior acrosomal region as well as in the postacrosomal region of the plasma membrane, with a very sharp boundary separating these regions from the almost sterol-free interposed equatorial segment [32]. Furthermore, the existence of a transmembranous barrier to lipid lateral diffusion in the equatorial region of the human sperm plasma membrane has recently been reported [33]. The high cholesterol content of sperm plasma membrane has been supposed to play a protective role against cell activation by inhibiting precocious, out of time triggering of acrosome reaction [34]. The conspicuous, progressive lowering of membrane cholesterol which takes place during capacitation [35], may then be rightly thought to represent the main way through which the spermatozoon acquires its biological responsiveness. The clear-cut relationships between Laurdan spectral properties and membrane structure and dynamics makes Laurdan spectroscopy a useful tool in investigating the correlations between the physical properties of the membrane environment and sperm functional events such as ion fluxes, receptor exposure, antigen recognition and fusional susceptibility both in normal and pathological conditions.

Acknowledgements

This work has been financially supported by grants (L.S.) from the Medical Faculty. The friendly advice and criticism of drs. Tiziana Parasassi and Settimio Grimaldi (Inst. Exptl. Med., C.N.R., Rome, Italy) are gratefully acknowledged.

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