Molecular Human Reproduction Vol.9, No.7 pp. 381–388, 2003 DOI: 10.1093/molehr/gag056

Idiopathic infertility: susceptibility of spermatozoa to *in-vitro* capacitation, in the presence and the absence of palmitylethanolamide (a homologue of anandamide), is strongly correlated with membrane polarity studied by Laurdan fluorescence

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Capacitation is a widely investigated process, which induces sperm plasma membrane changes resulting in its increased affinity for the zona pellucida. The fluorescent probe Laurdan, localized only within the plasma membrane of spermatozoa, is particularly useful to evaluate bilayer polarity in this part of the cell. According to a previous study, sperm membranes from oligozoospermic and some normozoospermic subjects (defined according to World Health Organization criteria), are characterized by low polarity (high Laurdan $exGP^{340}$), while the spermatozoa from the remaining normozoospermic men show a larger polarity (low $exGP^{340}$). In this paper, Laurdan was used to study membrane changes occurring during *in-vitro* capacitation, on sperm membranes from oligozoospermic and normozoospermic subjects. Results indicated that cells with high $exGP^{340}$ show a different susceptibility to Ca^{2+}-induced capacitation *in vitro*, as compared with cells with low $exGP^{340}$. Palmitylethanolamide, physiologically present in human reproductive tracts, affects the time-course of *in-vitro* capacitation, increasing the rate of this process only in the cells with a lower membrane polarity.

Key words: fluorescence/generalized polarization/Laurdan/male infertility/spermatozoa capacitation

Introduction

Defective sperm function is the most common cause of infertility, but it is difficult to evaluate and treat. Part of this difficulty is due to our incomplete understanding of the factors contributing to normal and abnormal function leading to male infertility. Moreover, some physical and functional characteristics of the sperm plasma membrane, largely involved in sperm fertilization capacity (Langlais *et al*., 1988; Hoshi *et al*., 1990), are not taken into account by standard semen analysis. In the first step of the fertilization process, sperm cells need to be activated in the female genital tract in a process called capacitation. Only capacitated sperm cells bind to the zona pellucida in a species-specific manner (Flesch and Gadella, 2000). An important role in fertilization processes, such as capacitation and the acrosomal reaction, is played by the sperm plasma membrane (Ladha, 1998; Flesch and Gadella, 2000).

One important aspect of the capacitation process is the remodelling of the glycocalyx on the plasma membrane which explains the dynamic alterations in lateral topology of the transmembrane proteins (Flesch and Gadella, 2000). Moreover, the glycocalyx may induce lateral polarity changes of lipids in the sperm plasma membrane, which in turn may induce topological rearrangements of freely diffusible transmembrane proteins. In freshly ejaculated sperm cells the glycolipids are concentrated in a sulphated form in the apical region (Flesch and Gadella, 2000). It is probable that glycolipids prevent the acrosome reaction by stabilizing the lipid lamellar bilayer of the plasma membrane and by impeding Ca^{2+} influx (Flesch and Gadella, 2000). However, during the capacitation process, seminolipids migrate into the equatorial region of the plasma membrane and become partly desulphated by arylsulphatases (Flesch and Gadella, 2000). Glycolipid migration is a prerequisite for the acrosome reaction and reflects an important capacitation event, which may be essential for mammalian fertilization. Since the capacitation process may modify membrane polarity (Gadella *et al*., 1995), the fluorescent membrane probe Laurdan (6-dodecanoyl-2-dimethylaminonaphtalene), which is particularly sensitive to polarity changes, could be a powerful technique to detect physico-chemical modifications occurring during the early stages of capacitation in sperm plasma membranes. The Laurdan molecule localizes at the hydrophobic–-hydrophilic interface of the lipid bilayer (glycerol backbone) and its spectral properties are due to the number and to the molecular dynamics of water dipoles in its environment, due to the effect of dipolar relaxation processes (Parasassi *et al*., 1991). This probe enables the detection of the coexistence of lipid gel and liquid-crystalline phases (phase heterogeneity) (Parasassi *et al*., 1991). It is particularly sensitive to membrane cholesterol content, which is known to modulate membrane lipid order and dynamics and to

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decrease membrane polarity (Parasassi *et al.*, 1994). As previously demonstrated (Palleschi and Silvestroni, 1996), Laurdan is localized only within the plasma membrane of spermatozoa, so it is particularly useful for this study. Moreover, its lateral and transbilayer partitioning could be considered uniform (Parasassi *et al.*, 1991). In a previous paper (Ambrosini *et al.*, 2001), we showed that sperm membranes from oligozoospermic and some normozoospermic subjects (defined according to World Health Organization, 1999), are characterized by low polarity (high Laurdan $exGP^{340}$), while the sperm from the remaining normozoospermic men show a larger polarity (low $exGP^{340}$). We suggested (Ambrosini *et al.*, 2001) for normozoospermic samples, with low polarity, similar compositional and physicochemical membrane alterations as in sperm cells of oligozoospermic patients.

The first aim of the present paper was to use Laurdan fluorescence properties to study *in-vitro* capacitation induced by Ca^{2+} on human sperm, in order to compare membrane changes occurring, during capacitation, in sperm membranes from oligozoospermic and normozoospermic subjects.

N-Acylethanolamides, in particular arachidonylethanolamide, palmitylethanolamide and other acylethanolamides (NAE), are present in mammalian reproductive tracts (testis and uterus), reviewed by Hansen *et al.* (2000). Accumulation of NAE in rat testis as a consequence of cadmium-induced stress has previously been established (Kondo *et al.*, 1998). Recent publications have demonstrated the presence of some NAE (arachidonylethanolamide, palmitylethanolamide and oleylethanolamide) in three human reproductive fluids (seminal plasma, oviductal and follicular fluids) (Burkman *et al.*, 2001; Schuel *et al.*, 2002). Their presence may imply sequential exposure of sperm cells to these agents, while moving from the male tract, up through the female reproductive tract. A possible modulatory role for at least some NAE during sperm transport, capacitation and fertilization has been suggested (Burkman *et al.*, 2001; Schuel *et al.*, 2002). In order to clarify the possible role of palmitylethanolamide on sperm physiological processes, the second aim of this paper was to study its effects on *in-vitro*-induced sperm capacitation processes, both in normozoospermic and oligozoospermic subjects.

Materials and methods

Reagents

Laurdan and Merocyanine 540 were purchased from Molecular Probes (USA). Before use, a stock solution of each probe was prepared in dimethylsulphoxide (DMSO). Dipalmitoylphosphatidylcholine (DPPC) was obtained from Avanti Polar (USA). All chemicals were purchased from Sigma Chemical Company (USA). Palmitylethanolamide (PEA) was synthesized as previously described (Ambrosini *et al.*, 1993a) and a stock solution was prepared in DMSO. The water used in this study was deionized with a Milli-Q water system by Millipore (USA).

Patients and semen samples

This study was approved by the institutional review board of the Polytechnic University of Ancona. Each patient gave informed consent. Fresh semen samples were obtained from 24 men (aged from 25 to 34 years) with primary infertility diagnosed after ⩾2 years of regular unprotected intercourse and with normal genital examination. The patients were undergoing sperm analysis at the Andrology Laboratory of the Division of Endocrinology, Umberto I Hospital, Polytechnic University of Ancona, Italy. Female partners were aged from 22 to 31 years; they had normal menstrual cycles with ovulatory activity, as assessed by progesterone concentrations on day 18–22 of the menstrual cycle. Semen samples were collected into plastic containers as previously described (Ambrosini *et al.*, 2001) and were allowed to liquefy at room temperature in the dark. Sperm count was determined by a Makler chamber; sperm cell motility and morphology were assessed according to World Health Organization (1999) standards. The same aliquot used for fluorescence measurements was also analysed by routine screening to evaluate any possible pathological conditions in semen characteristics (sperm culture, antisperm antibody detection by Mar-test); a urethral specimen was also collected for *Chlamydia* and *Mycoplasma urealyticum* detection. Sperm culture (*Chlamydia* and *M. urealyticum* detection) and antisperm antibodies were negative in all samples. Leukocytes and precursor cell concentrations were always $<1\times10^6$ cells/ml.

Laurdan-labelled liposomes

DPPC large unilamellar vesicles (LUVs) were prepared by extrusion through 0.1 μm polycarbonate filters at 55°C, using LiposoFast apparatus (Avestin, Inc., Canada) (MacDonald *et al.*, 1991). When present, the fluorescent probe Laurdan was added to DPPC to give a final probe/lipid Pi molar ratio of 1:1000, before the preparation of vesicles. Unlabelled LUVs were used to subtract background contribution to emission. LUVs were diluted to a final concentration of 0.3 mmol/l lipid Pi.

Sperm preparation and incubation

For each fluorescence experiment, samples with sperm cell concentration $\geqslant9\times10^6$/ml were selected. Samples were washed twice (centrifuged at 500 *g*, 10 min each) (Vijayakumar *et al.*, 1987) with washing buffer [KCl, 5.4 mmol/l; KH_2PO_4, 0.44 mmol/l; NaCl, 137 mmol/l; $NaHCO_3$, 4.2 mmol/l; $NaHPO_4$, 0.33 mmol/l; D-glucose, 5.6 mmol/l; pH 7.2 (as detailed in Gadella *et al.*, 1994)] in order to eliminate the seminal fluid coagulum. All samples were prepared to a final concentration of 10×10^6 cells/ml and maintained in the dark up to the time of use. Gadella *et al.* (1994) demonstrated that this buffer causes no deterioration of sperm over 6 h, considerably longer than the time period for our experiments. The washing buffer used to wash cells (Gadella *et al.*, 1994) was preincubated in a CO_2 incubator (5% CO_2 in air at 95% relative humidity) at 37°C for 16–18 h before use. For fluorescence measurements the sperm cells were resuspended in the same washing buffer at the same concentration (10×10^6 cells/ml).

Fluorescence measurements

Samples equilibrated for 20 min at 37°C in the dark were labelled with Laurdan as previously described (Palleschi and Silvestroni, 1996; Ambrosini *et al.*, 2001). According to Palleschi and Silvestroni (1996) no cytotoxicity or decrease of motility is induced by this probe on spermatozoa.

From the same specimen, two different samples were prepared: the first was used as control, the second was incubated with 5 μmol/l PEA for 15 min, before fluorescence measurements. Capacitation was induced by the injection of 2 mmol/l Ca^{2+} in the cuvette, according to the method of Gadella *et al.* (1994). Since PEA was dissolved in 1 μl of DMSO, control experiments were performed with the same volume of this solvent: no significant DMSO effects on Laurdan fluorescence and capacitation process were measured (data not shown).

Steady-state Laurdan fluorescence measurements were acquired both in the absence and in the presence of Ca^{2+}. Spectra acquisition was performed for 2 h after Ca^{2+} injection into the sample. An LS50 B PerkinElmer fluorometer (Perkin-Elmer, USA), equipped with a stirrer, was used for the steady-state fluorescence measurements. The cuvette temperature was controlled in a thermostable chamber by using a circulating water bath (HAAKE F3, Germany).

Both in cells and in DPPC LUVs, Laurdan emission spectra were recorded in the range from 420 to 550 nm, using both 340 and 410 nm excitation wavelengths. Fluorescence excitation spectra were obtained in the range from 300 to 420 nm, using both 435 and 490 nm emission wavelengths. Blank spectra were obtained with unlabelled cells or liposomes and were subtracted from the spectra of labelled cells or liposomes. For cells, a fixed temperature of 37°C was used for measurements. On the contrary, Laurdan fluorescence in DPPC LUVs was measured at temperatures above the main lipid phase transition, Tm, which is 41.2°C in DPPC LUVs, in our experimental conditions. From the spectroscopic data, Laurdan emission generalized polarization (emGP) spectra were derived by calculating the GP value, for each emission wavelength, by the following formula (Parasassi *et al.*, 1993):

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

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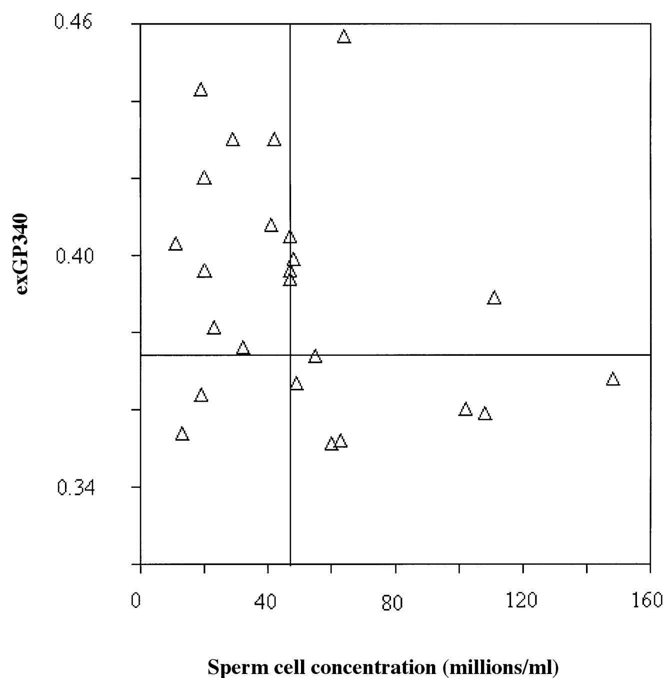

Figure 1. Laurdan $exGP^{340}$ values reported as function of sperm cell concentration.

where I_{410} and I_{340} are the intensities measured, at each emission wavelength, from 420 to 520 nm. These values are obtained by the fluorescence emission spectra recorded using fixed excitation wavelength of 410 and 340 nm, respectively. The excitation GP (exGP) spectra were constructed using the following formula (Parasassi *et al*., 1993):

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

where I_{435} and I_{490} are the intensities measured, at each excitation wavelength (from 320 to 420 nm), on the fluorescence excitation spectra obtained by fixed emission wavelength of 435 and 490 nm, respectively. The choice of 410, 340, 435 and 490 nm for GP calculations was based on the characteristic excitation and emission wavelength of pure and liquid-crystalline lipid phases, according to Parasassi *et al*. (1993).

Laurdan exGP and emGP spectra were calculated because previous work has demonstrated that they show characteristic patterns in the presence of pure or coexisting lipid phases (Parasassi *et al*., 1993) and in the presence of cholesterol (Parasassi *et al*., 1993). In fact, these spectra can determine whether domains of different composition and phase properties coexist in the plane of the membrane (membrane heterogeneity) (Parasassi *et al*., 1993). $ExGP^{340}$ was used as a parameter particularly sensitive to polarity (Palleschi and Silvestroni, 1996; Ambrosini *et al*., 2001; Antollini and Barrantes, 2002). Laurdan was chosen for its ability to detect changes in water penetration into the bilayer surface, that is strongly related to the membrane phase (Parasassi *et al*., 1991), but it is independent of PL polar headgroup and of pH in the range 4–10 (Parasassi *et al*., 1991). $ExGP^{340}$ can be calculated both by exGP spectra (choosing the value at 340 nm) or by the Laurdan emission spectrum ($ë_{ex}$ = 340 nm), used for calculation of the intensities at 435 and 490 nm. These wavelengths correspond to the characteristic emission wavelengths of Laurdan in pure phospholipid gel and liquid crystalline phases respectively. $ExGP^{340}$ (which is sensitive to the extent of water polar relaxation processes) gives a mathematically convenient and quantitative method to measure the Laurdan emission spectral shift. For phospholipids, characteristic $exGP^{340}$ values were found for the gel and liquid crystalline phases. However, the observation of a $exGP^{340}$ value intermediate between these values is not a final proof for the coexistence of separate phospholipid domains. In fact, the $exGP^{340}$ value was shown to be directly related to the increasing cholesterol content of a membrane (Parasassi *et al*., 1993). The Laurdan emGP and exGP spectra profile allows the determination of phase coexistence (Parasassi *et al*., 1993). Also these spectra are affected by the presence of cholesterol in a biological membrane (Parasassi *et al*., 1993).

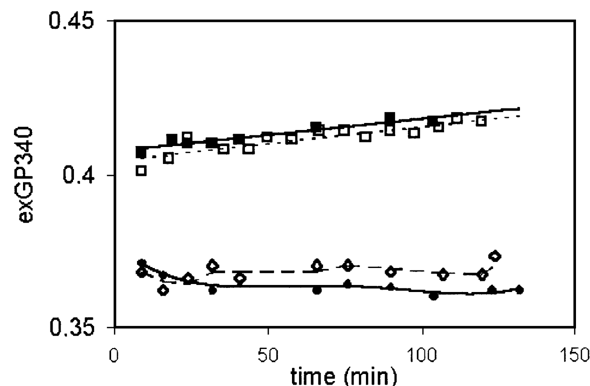

Figure 2. Time-course of $exGP^{340}$ changes during in-vitro capacitation of sperm cells from group I (diamonds) and group II (squares), both in the absence (filled symbols) and in the presence (empty symbols) of 5 µmol/l palmitylethanolamide.

Merocyanine 540 staining

To detect increases in plasma membrane lipid packing disorder, sperm cells were stained with 2.7 µmol/l Merocyanine 540 (MC540) (Flesch *et al*., 2001). Sperm cells (10×10^6 cells/ml) were suspended in the same buffer and with the same procedure used for Laurdan experiments.

Three samples with seminal parameters as group I (sperm cell concentration $\geqslant 49\times10^6$/ml and a mean $exGP^{340}$ of 0.365 ± 0.020) (with and without 2 mmol/l Ca^{2+}) were incubated for 10 min with MC540, before acquiring measurements at the chosen incubation time. Fluorescence emission spectra were obtained (using λex 540 nm) according to Baumann *et al*. (2000) and were recorded by the LS50 B Perkin Elmer fluorometer.

Results are expressed as means ± SD. The statistical significance of the data was evaluated by Student's *t*-test.

Results

We have characterized two groups of samples, presenting significantly different $exGP^{340}$ values ($P < 0.001$) and different sperm cell concentration (Figure 1). Of the subjects examined during this research, 83.3% can be classified into one of these two groups (20 subjects), while the remaining 16.7% (four subjects) did not follow this classification. Group I includes seven samples (29.2% of the patients) with sperm cell concentration $\geqslant 49\times10^6$/ml and an $exGP^{340}$ mean value of 0.365 ± 0.020. Group II (13 samples; 54.1% of the patients), with sperm cell concentration $<49\times10^6$/ml, are characterized by an $exGP^{340}$ mean value 0.400 ± 0.018. Figure 2 shows the $exGP^{340}$ behaviour in samples incubated with 2 mmol/l Ca^{2+}. It is evident that $exGP^{340}$ values (average of seven samples) decrease in group I and increase in group II (values are the average of 13 samples), as a function of Ca^{2+} incubation time (Figure 2). Table I presents the mean $exGP^{340}$ values obtained in these groups after 10 and 100 min of Ca^{2+} incubation. Group II data are significantly higher than group I at both incubation times ($P < 0.05$ at 10 min, and $P < 0.001$ at 100 min). The difference (Δ) between $exGP^{340}$ values measured in the two groups is larger after 100 min of Ca^{2+} incubation than after 10 min (0.057 and 0.036 respectively) (Table I).

During Ca^{2+} incubation, the $exGP^{340}$ values of group I are increased by PEA at almost every incubation time considered, while the $exGP^{340}$ values of group II are slightly decreased (Figure 2 and Table II). $ExGP^{340}$ values measured in the presence of PEA in group I are significantly different from those of group II.

With the aim of investigating the occurrence of domain segregation (heterogeneity) on the membrane plane during Ca^{2+} incubation, Laurdan exGP and emGP spectra were measured from 320 to 410 nm, and from 420 to 520 nm, respectively, as a function of ë to characterize

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the lipid phase of spermatozoa plasma membrane. The emGP and exGP spectra profiles present characteristic slopes in gel, liquid-crystalline lipid phases and in phase domain coexistence. In pure gel phase PLS, both exGP and emGP spectra show only little variation with excitation and emission wavelength. In pure liquid-crystalline phase the exGP spectrum shows decreasing values with increasing excitation wavelength, while emGP spectrum shows increasing values as the emission wavelength increases. The opposite behavior is obtained in the presence of liquid phase separation (Parasassi *et al.*, 1993). In order to quantify the slope changes, we have used the relative values of $exGP^{340}$ and $exGP^{410}$, measured on exGP spectra (at 340 and 410 nm) (Figure 3A and B respectively) and $emGP^{435}$ and $emGP^{490}$, measured on emGP spectra (at 435 and 490 nm) (Figure 3C and D respectively). In agreement with previous results (Ambrosini *et al.*, 2001), all samples gave a shape characteristic of an ordered liquid-crystalline phase, since $exGP^{340} > exGP^{410}$ and $emGP^{435} < emGP^{490}$. In the absence of Ca^{2+}(Figure 3, white bars), PEA does not significantly modify exGP and emGP values of either group. In group I: (i) a significant decrease in $exGP^{410}$ only after 2 h Ca^{2+} incubation (from 0.260 ± 0.030, in the absence of Ca^{2+}, to 0.197 ± 0.030, $P < 0.005$, for samples without PEA, and from 0.250 ± 0.030 to 0.190 ± 0.031, $P < 0.005$, in the presence of PEA) (Figure 3, $exGP^{410}$, black bars); (ii) a significant decrease of $emGP^{435}$ after 2 h incubation (from –0.640 ± 0,030 in the absence of Ca^{2+}, to – 0.690 ± 0.030, $P < 0.010$, for samples without PEA; and from –0.670 ± 0.030 to –0.708 ± 0.035, $P < 0.05$, in the presence of PEA) (Figure 3, $emGP^{435}$, black bars). GP measured at 340 nm on exGP spectra ($exGP^{340}$) and at 490 nm on emGP spectra ($emGP^{490}$) (Figure 3, $exGP^{340}$ and $emGP^{490}$ respectively) are not significantly changed by Ca^{2+}, in all samples considered. These data indicate that membrane heterogeneity is significantly modified only after 2 h Ca^{2+} incubation, both in the absence and in the presence of PEA.

Table I. $exGP^{340}$ values calculated in groups I and II, both in the presence and in the absence of 5 µmol/l palmitylethanolamide (PEA)

Time (min)	Group I	Group II	Δ
No PEA			
10	0.371 ± 0.009	0.407 ± 0.027**	0.036
100	0.360 ± 0.014	0.417 ± 0.026***	0.057
With PEA			
10	0.363 ± 0.024	0.399 ± 0.024*	0.036
100	0.369 ± 0.021	0.410 ± 0.028*	0.041

Δ represents the difference between the two groups. Data are presented as means ± SD of values measured after 10 min and 100 min incubation with Ca^{2+}.
*$P < 0.01$, **$P < 0.005$, ***$P < 0.001$ significance group I versus group II.

The GP values acquired on group II samples, in the absence of PEA, show significant differences in $exGP^{410}$ and $emGP^{435}$, only after 2 h Ca^{2+} incubation (Figure 3, $exGP^{410}$ and $emGP^{435}$ respectively, black bars). In fact, $exGP^{410}$ ranges from 0.277 ± 0.025, in the absence of Ca^{2+}, to 0.255 ± 0.022, after 1 h incubation (Figure 3, $exGP^{410}$, grey bars), and 0.208 ± 0.025, $P < 0.001$, at 2 h (Figure 3 $exGP^{410}$, black bars). $ExGP^{435}$ ranges from –0.669 ± 0.030, in the absence of Ca^{2+}, to –0.690 ± 0.035, after 1 h Ca^{2+} incubation (Figure 3 $exGP^{435}$, grey bars), and –0.731 ± 0.030, $P < 0.001$, after 2 h (Figure 3 $exGP^{435}$, black bars). In the presence of PEA, Ca^{2+} causes significant decreases of $exGP^{410}$ and $emGP^{435}$, with respect to samples without Ca^{2+}, both after 1 and 2 h Ca^{2+} incubation. In fact, $exGP^{410}$ ranges from 0.291 ± 0.036, in the absence of Ca^{2+}, to 0.234 ± 0.035, $P < 0.001$, after 1 h Ca^{2+} incubation (Figure 3 $exGP^{410}$, grey bars), and 0.195 ± 0.040, $P < 0.001$, at 2 h (Figure 3 $exGP^{410}$, black bars). $EmGP^{435}$ ranges from –0.644 ± 0.025 in the absence of Ca^{2+}, to –0.736 ± 0.030, $P < 0.001$,

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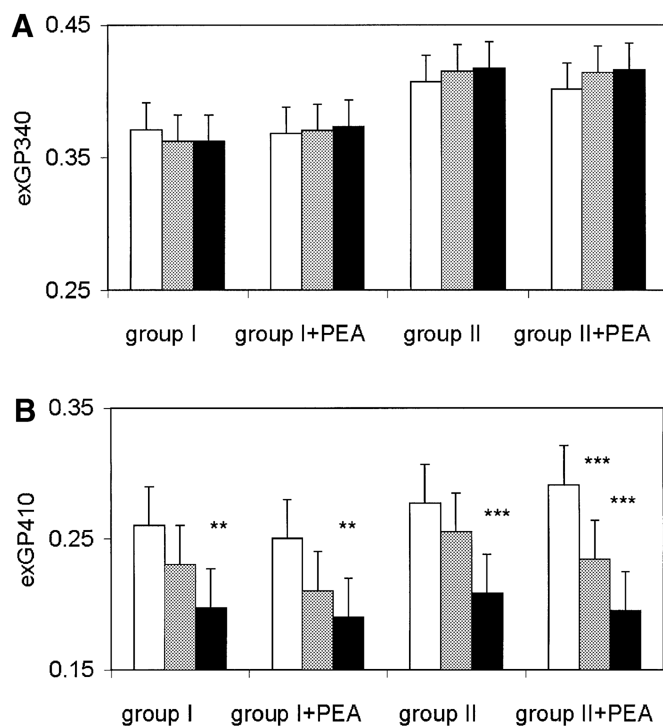

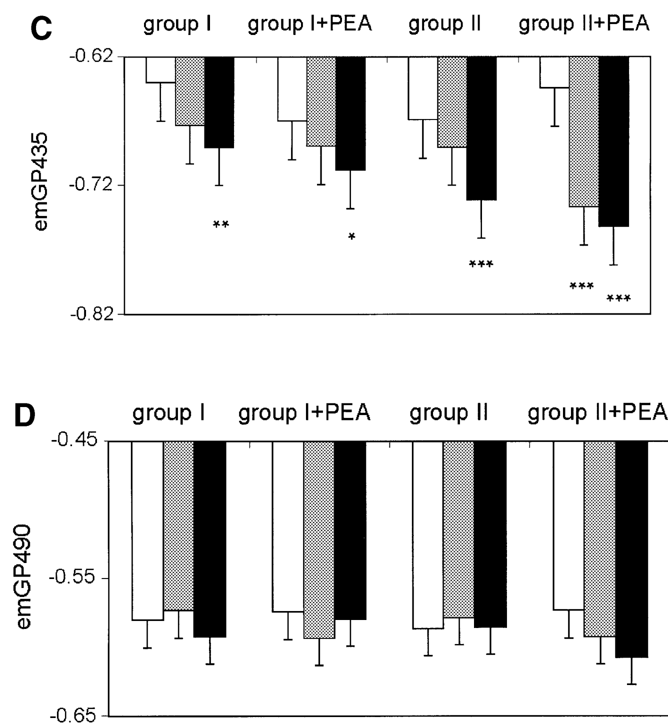

Figure 3. Effect of Ca^{2+} incubation on Laurdan GP spectra recorded in group I and group II sperm cells: exGP and emGP mean values measured at 340 nm ($exGP^{340}$) (**A**), 410 nm ($exGP^{410}$) (**B**), 435 nm ($emGP^{435}$) (**C**) and 490 nm ($emGP^{490}$) (**D**). Values measured in the absence of Ca^{2+} (white bars) are compared with values measured after 1 h (grey bars) and 2 h (black bars) Ca^{2+} incubation. SD are given (with error bars). *$P < 0.05$; **$P < 0.005$; ***$P < 0.001$. PEA = palmitylethanolamide.

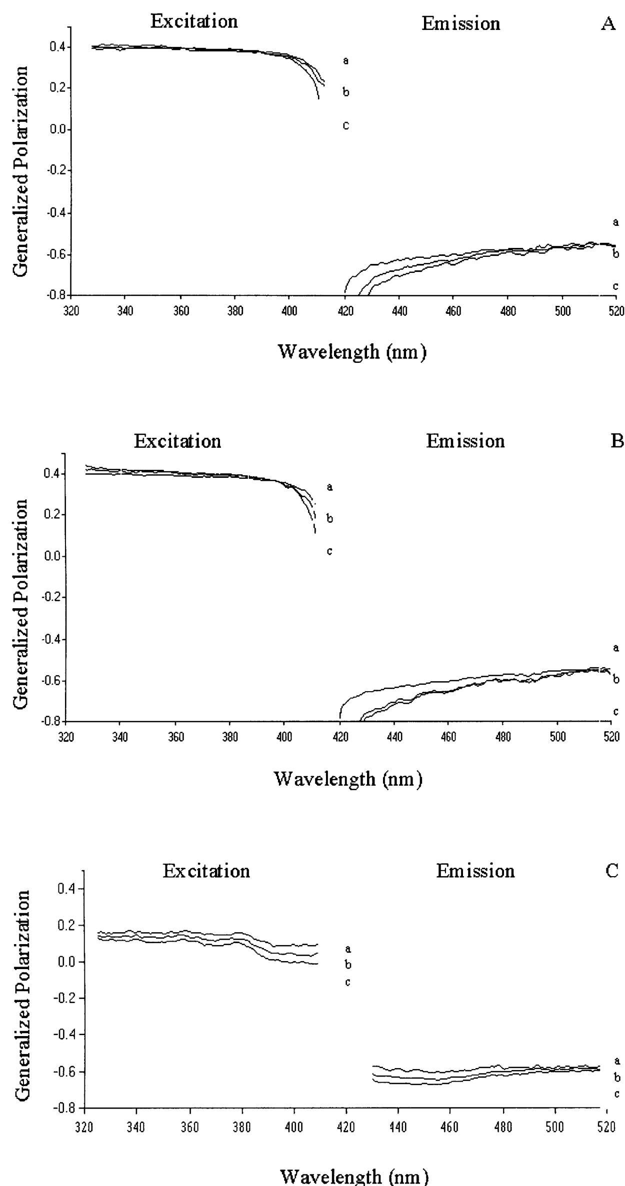

Figure 4. (**A**) Laurdan GP spectra in sperm cell membrane (group II) in the absence of (a) and after 1 h (b) and 2 h (c) incubation with Ca^{2+}. (**B**) Laurdan GP spectra in sperm cell membrane (group II), incubated with PEA, in the absence of (a) and after 1 h (b) and 2 h (c) incubation with Ca^{2+}. (**C**) Laurdan GP spectra in dipalmitoylphospathidylcholine large unilamellar vesicles at 42°C (a), 44°C(b) and 46°C (c).

after 1 h Ca^{2+} incubation (Figure 3 $emGP^{435}$, grey bars), and -0.752 ± 0.040, $P < 0.001$, at 2 h (Figure 3 $emGP^{435}$, black bars). In group II, $exGP^{340}$ and $emGP^{490}$ do not change significantly in the presence or in the absence of PEA following Ca^{2+} incubation (Figure 3 $exGP^{340}$ and $emGP^{490}$ respectively). Data indicate also that in group II membrane heterogeneity is significantly modified only after 2 h Ca^{2+} incubation. In this case PEA significantly affects the time-course of membrane heterogeneity modification, which becomes evident after 1 h.

In order to elucidate the origin of these spectral changes, we studied the Laurdan GP spectra profile in DPPC LUVs. Figure 4 shows the comparison between Laurdan exGP and emGP spectra acquired in group II sperm cells, both in the absence (Figure 4A) and in the presence (Figure 4B) of PEA (curves presented in the figure are the average of the curves obtained by all the samples of the group), and the same spectra obtained in DPPC LUV (Figure 4C). The effect of temperature increase on GP spectra behaviour in the DPPC liquid-crystalline phase is also shown. From Figure 4C, it is evident that, in DPPC LUV, temperature-induced larger decreases are measurable on $exGP^{410}$ and $emGP^{435}$, while smaller modifications are shown by $exGP^{340}$ and $emGP^{490}$.

In the absence of PEA, the effect induced by Ca^{2+} on Laurdan GP spectra, after 1 and after 2 h of incubation, is more evident on the red edge of the excitation band and on the blue edge of the emission band. This effect is evident in both groups. Comparison with experiments in DPPC LUV shows that an increase in temperature (inducing a larger disorganization of the lipid bilayer in the liquid crystalline phase) produces similar decreases on exGP and emGP spectra.

Laurdan GP spectra, obtained after 1 and 2 h of incubation with the cation and PEA, present the same slope (see Figure 4B). On the contrary, in the sample without PEA, the slope of 2 h incubation spectra are significantly increased with respect to that obtained after 1 h incubation (Figure 4A). This result indicates that the presence of PEA increases the rate of the capacitation process measured by Laurdan fluorescence.

Of the 16.7% of samples (four subjects) that do not follow the previous classification into group I or II: two samples are characterized by $exGP^{340}$ in the range of group I, but $<49\times10^6$ cells/ml, while the opposite characteristics are shown by two other samples. It is noteworthy that, both in the presence and in the absence of PEA, the behaviour of Ca^{2+}-induced capacitation *in vitro* in these samples is similar to the time-course of this process in the groups with similar $exGP^{340}$ (data not shown).

In order to verify that, in our experimental conditions, $exGP^{340}$ behaviour is correlated with the capacitation process, we have performed control experiments with MC540, a lipophilic fluorochrome. It was previously shown that the fluorescence emission intensity of this probe is largely increased during the capacitation (Gadella *et al*., 2002). The fluorescence features of this probe are related to its affinity for a given plasma membrane leaflet, which depends on the degree of lipid structural disorder in the leaflet (Harrison and Miller, 2000). It was demonstrated that the increased fluorescence emission measured in spermatozoa is due to the membrane destabilization occurring during capacitation, while it is apoptosis-unrelated (Gadella *et al*., 2002). Figure 5B shows MC540 fluorescence emission intensity as a function of incubation time, in the presence and absence of Ca^{2+}: it is evident that the cation induces a time-dependent significant increase in MC540 intensity, indicating a decreased packing order on the membrane. Results presented are the average of three different experiments performed on subjects with normal semen parameters (group I) (sperm cell concentration $\geqslant 49\times10^6$/ml). Laurdan experiments were performed on the same samples (Figure 5A). The inverse correlation between MC540 fluorescence emission increases and $exGP^{340}$ value decreases was established ($r = 0.9$, $P < 0.05$) on the samples incubated with Ca^{2+}.

Discussion

Capacitation is a widely investigated but very complicated process, which induces sperm plasma membrane changes resulting in its increased affinity for the zona pellucida (Flesch and Gadella, 2000). One of the complications in capacitation research is the difficulty of discriminating between induced changes at the plasma membrane and those in the remaining part of the sperm cell (Flesch and Gadella, 2000).

Capacitation can be induced *in vitro* by different methods, with some different effects at the membrane level (Gadella *et al*., 1994).

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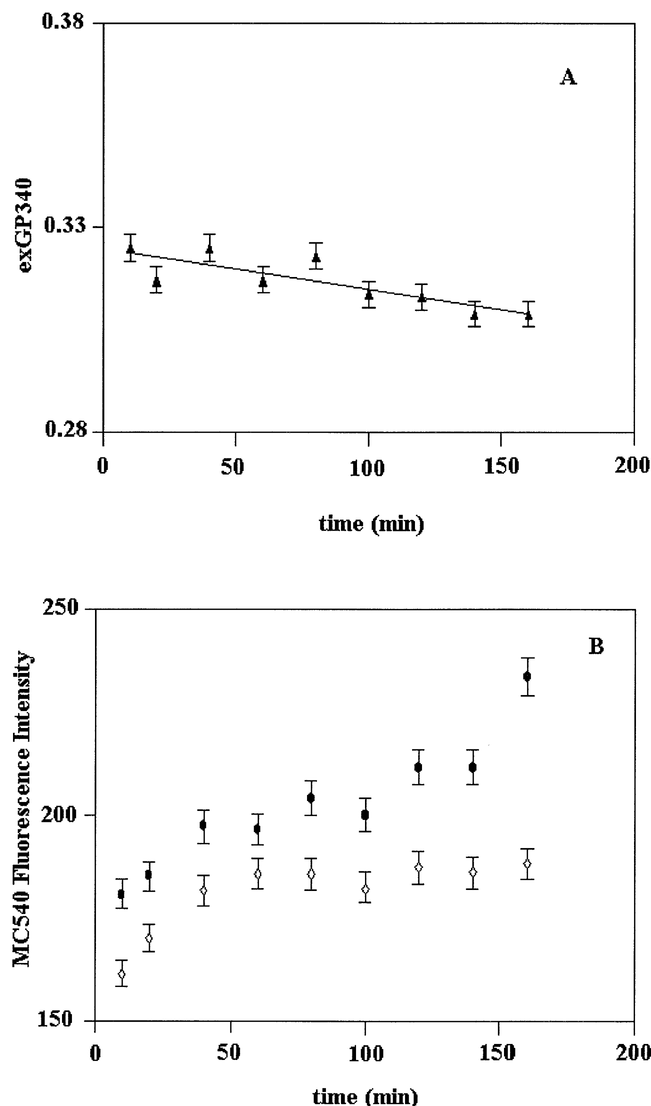

Figure 5. Laurdan exGP[340] measured in sperm cells incubated with 2 mmol/l Ca^{2+} (**A**); Merocyanine 540 fluorescence emission intensity (λem 585 nm) measured in the same sperm cells, in the presence (filled) and in the absence (empty) of 2 mmol/l Ca^{2+} (**B**). Results are presented as a function of time of incubation with Ca^{2+}. Data are the mean ± SD of three different experiments.

Gadella *et al.* (1995) demonstrated that, in the absence of A23187 ionophore, 2 mmol/l Ca^{2+} or bovine serum albumin (BSA) affect the sperm membrane in a similar way, because they induce the migration of glycolipids from the acrosome to the equatorial segment. After 2 h of incubation with extracellular Ca^{2+}, this migration took place in ~85% of sperm cells (Gadella *et al.*, 1995). It was suggested that glycolipid migration induces fluidity changes in the sperm plasma membrane, due to compositional modifications of membrane lipid domains (Gadella *et al.*, 1995); these processes should induce a facilitated acrosomal exocytosis.

As demonstrated elsewhere (Palleschi and Silvestroni, 1996), Laurdan is localized only within the plasma membrane of sperm, so it is particularly useful to study physico-chemical changes in this part of the cell. Previous studies by Laurdan fluorescence have shown a liquid-crystalline lipid phase (Palleschi and Silvestroni, 1996; Ambrosini *et al.*, 2001) and the lack of thermotropic phase transitions in the plasma membrane of living human sperm cells (Palleschi and Silvestroni, 1996). This behaviour could be due to the large cholesterol/phospholipid (chol/PL) ratio of the sperm membranes. In fact, cholesterol represents 40–50% of total lipids of human sperm plasma membranes (Ladha, 1998; Flesch and Gadella, 2000).

It has been demonstrated that chol/PL ratio is an important chemical index of membrane fluidity (Ladha, 1998). In rat sperm, decreases in this ratio correlate with an increase in local polarity, fluidity and molecular disordering (Kumar, 1993). Moreover, a lower chol/PL ratio in human sperm was shown to correlate with a faster capacitation time (Hoshi *et al.*, 1990).

It has previously been shown that plasma membranes of sperm from oligozoospermic patients have an increased chol/PL ratio (Huacuja *et al.*, 1981). It was suggested that the more rigid lipophilic membrane domains of these patients could be a cause of their infertility problems (Ladha, 1998). Furthermore, it was recently demonstrated that an abnormally low membrane fluidity exists in swim-up selected sperm, from men with pathological semen characteristics (Force *et al.*, 2001). It was hypothesized that this low fluidity could explain the failure of fertilization by sperm of these patients (Force *et al.*, 2001). In a previous study (Ambrosini *et al.*, 2001) we suggested that the decreased polarity (high Laurdan exGP[340] values) measured in plasma membranes of oligozoospermic patients could be related to their high chol/PL ratio (Huacuja *et al.*, 1981). It was proposed that the normozoospermic samples (following World Health Organization criteria) which present similarly high exGP[340] values could be characterized by a similarly high chol/PL ratio, most likely due to incomplete and/or imperfect sperm maturation processes (Ambrosini *et al.*, 2001). In that study (Ambrosini *et al.*, 2001), we proposed the Laurdan exGP[340] parameter as a useful criterion to evaluate the fertilizing capacity of human sperm in some cases of idiopathic infertility.

In the present study, we confirmed that within subjects with idiopathic infertility the sperm plasma membrane shows different degrees of polarity, as indicated by Laurdan exGP[340] data. Moreover, these subjects show a different susceptibility to Ca^{2+}-induced capacitation *in vitro*. In line with our previous work (Ambrosini *et al.*, 2001), the subjects studied can be divided in two groups: (i) group I, characterized by smaller exGP[340] sperm cell concentration $\geqslant 49\times 10^6$/ml and an increase of membrane polarity as a consequence of incubation with Ca^{2+}; (ii) group II, with higher exGP[340], sperm cell concentration $<49\times 10^6$/ml, and a Ca^{2+}-induced decrease of membrane polarity. These results confirm that some normozoospermic men (according to World Health Organization criteria) show similar high exGP[340] values as oligozoospermic patients (Ambrosini *et al.*, 2001). The differences with the previous work (Ambrosini *et al.*, 2001) are likely to be attributed to the changes in the experimental conditions used (different buffer), which are necessary to induce capacitation. Our results indicate that Ca^{2+}-induced capacitation *in vitro* increases the differences between the two groups (when referred to the exGP[340] values) indicating an increased difference in the physico-chemical properties of the plasma membrane, in particular in membrane polarity. The physico-chemical properties of the lipid bilayer are known to affect many important physiological membrane functions and/or the proper activity of membrane proteins. Bilayer polarity is known to affect important processes such as membrane fusion or the binding of substances to membrane bilayers (Epand and Kraayenhof, 1999). Moreover, it is known that an increased local polarity and fluidity of the sperm membrane is associated with the acquisition of its fertilizing ability (Sinha *et al.*, 1994). Our data could indicate that subjects classified in group II, and characterized by a decreased membrane polarity (high Laurdan exGP[340] value), can present physiologically altered plasma membrane capacitation processes which could be related to their infertility.

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The correlation of Laurdan exGP^{340} behaviour with capacitation was confirmed by comparison with MC540 fluorescence. Merocyanine fluorescence increase is related to the decreased packing order of phospholipids of the outer leaflet occurring during the capacitation process (Gadella and Harrison, 2002). In our experimental conditions MC540 fluorescence increase follows the same time pattern as Laurdan exGP^{340} decrease, suggesting that the two probes are signalling the same event at the membrane level.

A possible modulatory role for some NAE, such as PEA, was proposed by Burkman *et al*. (2001) during sperm transport, capacitation and fertilization *in vivo* in human reproductive tracts. This possibility was suggested because the presence of these molecules was demonstrated in reproductive human organs and fluids, although their biological role is not clear. The NAE of the human reproductive tract are present in many cells and tissues (Schmid *et al*., 1990; Hansen *et al*., 2000). They are accumulated in stress conditions (Schmid *et al*., 1990), e.g. a large increase in PEA content was observed in testis of cadmium chloride-treated rats (Kondo *et al*., 1998). Some NAE are recognized by cannabinoid (CB) receptors and present cannabimimetic properties (De Petrocellis *et al*., 2000). This is the origin of the name 'endocannabinoids' used for these molecules. CB receptors are not always involved in NAE physiological functions (Hansen *et al*., 2000). It was speculated that saturated and monounsaturated NAE, such as PEA, could have some important functions: they are anti-inflammatory (Schmid *et al*., 1990), neuroprotective, analgesic (De Petrocellis *et al*., 2000), and can have cytoprotective effects (Skaper *et al*., 1996; Gulaya *et al*., 1998; Hansen *et al*., 2000). Although a mechanism involving the interaction of PEA with specific receptors cannot be excluded, however, the lipid nature of this molecule could suggest that, at least part of its biological functions could be related to physical interactions with the lipid bilayer (Schmid *et al*., 1990; Ambrosini *et al*., 1993a;b).

In the present work, the possible role of PEA on sperm functions was studied on the *in-vitro* capacitation process. The presence of PEA increases the time-course of Ca^{2+}-induced capacitation in group II, as evident by spectra shown in Figure 4. The same behaviour is not evident in group I. Although the molecular mechanisms of these PEA effects are not known, our results support the Burkman hypothesis on a possible modulatory role of NAE on the fertilization process. The capacitation process is a preparatory process of membrane destabilization which takes place prior to sperm encountering the oocyte (Gadella and Harrison, 2002). It is reasonable to hypothesize that the PEA interaction with the membrane could affect the activity of membrane enzymes which are involved in the capacitation process. This activity modulation could be performed by affecting the physico-chemical properties of the lipid bilayer, as suggested by previous studies (Ambrosini *et al*., 1993a;b) or by a direct interaction with the enzymes. The lipid nature of PEA suggests that its effects could be performed, at least partially, through physical interactions with the lipid part of the membrane. Although the effects of PEA incubation with sperm on Laurdan exGP^{340} are not significant, the possible preferential localization of NAE in specific membrane domains, with different lipid composition, could give rise to microenvironments with different structural and physico-chemical features (Ambrosini *et al*., 1993a;b), which could modify the activity of specific membrane enzymes. Our data suggest that the PEA effects could be related to the different lipid compositional features hypothesized for the sperm plasma membrane characterized by different polarity (Ambrosini *et al*., 2001). The evidence that PEA, in some cases, can increase the rate of capacitation *in vitro*, could suggest the possible pharmacological use of this molecule.

Acknowledgements

This paper was supported by M.I.U.R. 60% to G.Z.

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Submitted on April 4, 2002; resubmitted on February 11, 2003; accepted on March 3, 2003

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