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Idiopathic Infertility: Effect of Palmitoylethanolamide (a Homologue of Anandamide) on Hyperactivated Sperm Cell Motility and Ca^{2+} Influx

ANNARINA AMBROSINI,* GIOVANNA ZOLESE,* SIMONA AMBROSI,* ENRICO BERTOLI,* FRANCO MANTERO,† MARCO BOSCARO,‡ AND GIANCARLO BALERCIA‡

*From the *Institute of Biochemistry, Polytechnic University of Marche, Ancona, Italy; †Endocrinology, Department of Medical and Surgical Sciences, University of Padua, Padua, Italy; and ‡Endocrinology, Department of Internal Medicine, Umberto I Hospital, Polytechnic University of Marche, Ancona, Italy.*

ABSTRACT: The goal of this study was to examine the effect of palmitoylethanolamide (PEA) on the capacitation process and hyperactivated motility (HA) in idiopathic infertile men. Our data show the effect of PEA on the kinematic parameters of sperm cells from idiopathic infertile men during the capacitation of spermatozoa in vitro, both in the presence and absence of 2.5 nM PEA, a molecule physiologically present in human reproductive tracts. Two groups of sperm cells were identified. In group I ($36 \pm 14 \times 10^6$ cells/mL), PEA significantly increased some motility parameters and HA during capacitation. In group II ($58 \pm 18 \times 10^6$ cells/mL), PEA did not significantly modify motility parameters and HA. Fura 2 AM (acetoxymethyl ester derivative of fura 2) measurements demonstrated that PEA increased external Ca^{2+} influx (which modulates HA) in group I, while no change was measured in group II. In conclusion, our data indicated that PEA modulated certain physiological sperm functions that are involved in fertilization; in particular, we showed that PEA modulated for HA in men with low sperm kinematic parameters.

Key words: Kinematic parameters, hyperactivated motility, fura 2 AM.

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N-Acylethanolamides (NAEs) (amides of ethanolamine with long-chain fatty acids) are defined as endogenously generated lipid-signaling molecules and are widely distributed in a variety of plant, invertebrate, and mammalian tissues (Lambert et al, 2002; Schmid and Berdyshev, 2002).

The interest in NAEs is due to the quite recent discovery that some of these molecules, the derivatives of polyunsaturated fatty acids, can bind to and activate cannabinoid receptors (CB1 and CB2) (De Petrocellis et al, 2000). These NAEs are known as endocannabinoids, and the most studied of this family is the arachidonoylethanolamide derivative (AEA, anandamide). Anandamide occurs in trace amounts in almost all vertebrate cells and tissues (Schmid et al, 2002) and are always accompanied by NAEs characterized by saturated and monounsaturated acyl chains, such as palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) (Schmid et al, 2002). The possible binding of PEA to cannabinoid receptors is being investigated. It is known that PEA does not bind to CB1, and the hypothesis that it is a ligand of CB2 receptors is questioned (Lambert et al, 2002). OEA does not activate currently known cannabinoid receptors.

NAEs have also been isolated in mammalian reproductive tracts (testis and uterus) (Hansen et al, 2000) and in human reproductive fluids (seminal plasma and oviductal and follicular fluids) (Schuel et al, 2002a). In particular, the presence of AEA, PEA, and OEA has been demonstrated in these tissues. Their presence may imply a sequential exposure of sperm cells to these agents during their journey from the male and through the female reproductive tract. So a modulatory role for at least some NAEs during human sperm transport, capacitation, and fertilization has been suggested (Schuel et al, 2002a).

In human sperm, AEA signaling seems to be involved in processes of fertilization (Schuel et al, 2002b). However, the effect of the other NAEs that are present in the human reproductive tract must be studied. It has been suggested that PEA and OEA act as ''entourage compounds,'' modulating the effect of AEA (Schuel et al, 2002a). Moreover, PEA and OEA are potent anti-inflammatory, antioxidant, and antimicrobial agents. These findings suggest that NAEs can perform multiple roles in reproductive tract fluids by modulating sperm capacitation, regulating reproductive tract function, protecting against infection, and maintaining sperm viability (Schuel et al, 2002a).

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Correspondence to: Prof Giovanna Zolese, Istituto di Biochimica, Facoltà di Medicina, Via Ranieri, I-60100 Ancona, Italy (e-mail: g.zolese@univpm.it).

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Sperm capacitation is a poorly understood process that has been associated with modifications in sperm plasma membrane composition and fluidity, alterations in intracellular ion concentrations, and changes in oxidative metabolism (Marin-Briggiler et al, 2002). As a result of capacitation, spermatozoa develop hyperactivated motility (HA) and can undergo acrosome reactions in response to certain stimuli, such as the homologous zona pellucida, progesterone, and follicular fluid (Yanagimachi, 1994; Marin-Briggiler et al, 2002). HA is linked to the process of capacitation (Katz et al, 1989) and has been proposed as one of the criteria for evaluating the fertilizing potential of human spermatozoa (Chan et al, 1992; Johnston et al, 1994; Suarez, 1996; Kay and Robertson, 1998). It is known that an obligatory increase in free cytosolic calcium ($[Ca^{2+}]_i$) plays a major role in regulating HA (Quill et al, 2003). It was previously demonstrated that spermatozoa from subjects with idiopathic infertility exhibit lower HA than spermatozoa from fertile men (Peedicayil et al, 1997). In a previous work (Ambrosini et al, 2003), we demonstrated that PEA affects Ca^{2+}-induced sperm capacitation in vitro, and its effect is strongly correlated with sperm membrane physical properties in cases of idiopathic infertility.

The goal of this study was to investigate, in idiopathic infertile men, the possible modulatory action of PEA on sperm kinematic parameters and on the development of HA.

This study could elucidate the effect that the molecular mechanism responsible for the modulation of PEA activity has on the process of fertilization. The alteration of this effect could be the cause, at least in part, of idiopathic infertility.

To determine whether a PEA effect occurs via increased intracellular calcium levels, we used as a fluorescent probe the calcium indicator fura 2 AM (acetoxymethyl ester derivative of fura 2) (Grynkiewicz et al, 1985).

Materials and Methods

Reagents and Culture Media

Fura 2 AM was purchased from Molecular Probes Inc (Eugene, Ore). Before use, a stock solution of the probe was prepared in dimethylsulfoxide (DMSO). A stock solution of PEA was also prepared in DMSO. PEA was kindly provided by Prof M. Wozniak, Gdansk, Poland. The medium used in this study (Medi-Cult, Hopkinton, Mass) is based on the Earle Balanced Salt Solution (EBSS) buffered with 25 mM HEPES, and it is routinely used to induce sperm capacitation for assisted reproduction. It consisted of the following: 116 mM NaCl, 1 mM NaH_2PO_4, 5.37 mM KCl, 26 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.5 mM D-glucose, a concentration of both streptomycin and penicillin of 50 μg/mL, phenol red, and a percent concentration of human serum albumin not higher than 0.3% (Sil-Select Stock; FertilPro N.V., Beernem, Belgium). Higher albumin levels sequester cannabinoids and reduce the binding of ligands to sperm (Schuel et al, 2002b).

Patients and Semen Samples

Patient Selection—Nineteen patients (aged 25 to 34 years) affected by primary idiopathic infertility were enrolled in the study. The subjects were selected at the Andrology Unit of the Endocrinology Division, Umberto I Hospital, Polytechnic University of Marche, Ancona, Italy. The criteria for patient selection were chosen to be similar to those used in a previous work on the effect of PEA on idiophatic infertility (Ambrosini et al, 2003). Additionally, 4 samples from subjects of proven fertility were analyzed to compare the magnitude of their response with respect to those from the patients affected by primary idiopathic infertility. All of the subjects with a history of infertility after at least 2 years of regular unprotected intercourse underwent medical screening, including a clinical examination. Testicular volume was evaluated in each patient using a Prader orchidometer. To achieve a complete diagnosis, the following investigations were also performed:

1) Semen analysis,
2) Mar-test (SperMar test; Diasint, Florence, Italy) for anti-spermatozoa antibodies,
3) Sperm culture and a collection of urethral specimens to facilitate the detection of *Chlamydia* and *Mycoplasma ureoliticum*,
4) Follicle-stimulating hormone, luteinizing hormone, testosterone, estradiol (E2), and prolactin (PRL) assays, using commercial radioimmunoassay kits, and,
5) Testicular, prostatic, and seminal vesicle ultrasonography and echo-color Doppler of venous spermatic plexus, to facilitate the detection of anatomic abnormalities and varicocele.

No female-related factor was apparently involved in sterility, since all partners (aged 22 to 31 years) were ovulating regularly, as formally proven by luteal-phase progesterone levels; no abnormal fallopian tube anatomy was detected after hysterosalpingography.

The study was approved by the Institutional Review Board of the Polytechnic University of Marche. All subjects provided their written consent.

Eligibility Criteria—The following criteria were adopted for patient eligibility: 1) a sperm count greater than 20×10^6/mL and normal sperm morphology greater than 30%, according to World Health Organization strict criteria (1999); class a + b + c motility according to World Health Organization standards (1999) that were considered a percentage of motility for the purposes of the present study; 2) seminal white blood cell counts of less than 1×10^6/mL, negative sperm culture, and negative *Chlamydia* and *M ureoliticum* detection; 3) normal serum levels of gonadotropins, testosterone, E2, and PRL; 4) the absence of infectious genital diseases as well as anatomic abnormalities of the genital tract, including varicocele, and of anti-spermatozoa Ab; 5) the absence of systemic diseases or treatment with other drugs within the 3 months before enrollment in the present

study; and 6) the absence of smoking, alcohol, or drug addiction and occupational chemical exposure.

Semen Analysis—Semen samples were collected as previously described (Ambrosini et al, 2001), allowed to liquefy at room temperature, and processed within 1 hour after ejaculation. Semen quality was assessed by the same biologist in terms of sperm concentration and motility, in accordance with World Health Organization criteria (1999). The sperm count was determined with a Makler chamber. Motile spermatozoa were assessed by phase-contrast microscopy (10 μL of semen was delivered onto a glass slide and covered with a 22 × 22-mm coverslip) and graded as follows: classes a and b, fast and weak forward motility; class c, nonprogressive motility; and class d, immobile spermatozoa. Sperm morphology was evaluated on smears of seminal fluid, stained by the Giemsa method, and observed by oil immersion light microscopy.

Computer-assisted semen analysis (CASA) for the sperm cell motility assay was additionally performed, as previously reported (Ambrosini et al, 2001; Balercia et al, 2004). One semen aliquot (3 μL) was placed in a 20-μm-deep chamber. Two VU chambers with a depth of 20 μm (Conception Technologies, La Jolla, Calif) were loaded, and 6 different fields per chamber were randomly examined. At least 200 sperm for each field of the chamber were scored. Percentages of motile sperm and movement characteristics were analyzed using an automated analyzer at 37°C (CellTrack VP110; Motion Analysis Corporation, Palo Alto, Calif). Sperm velocity and kinematic characteristics were evaluated only for motile sperm and expressed as mean values, using curvilinear velocity (VCL) and straight progressive velocity (VSL).

Sperm Suspension Preparation—The specimens were allowed to liquefy for 30 to 60 minutes at 37°C in a slide warmer. Motile spermatozoa were selected by centrifugation through a 2-step Percoll gradient, which was prepared from Sil-Select Stock (FertilPro). Sil-Select Stock consists of silane-coated colloidal silica particles suspended in the medium (HEPES-buffered EBSS). Stock solutions of PEA (0.001 M in DMSO) were stored at −20°C and diluted to the proper concentration immediately before each experiment. PEA was injected directly into the medium. DMSO was always 0.2% (vol/vol). The control experiments were performed with the same volume of this solvent. Preliminary experiments showed that this concentration of DMSO had no significant effects on the kinematic parameters of sperm cells (data not shown). For each subject, 2 Percoll gradients were prepared: one containing 2.5 nM PEA and one containing DMSO. The aliquots of semen (up to 2 mL, 40 million cells for each sample) were layered over the upper step of the Percoll gradient and centrifuged for 12 minutes at 350 × *g*. The pellet was diluted in the medium and washed 2 times at 300 × *g* for 10 minutes. At the end of centrifugation, the supernatant was removed, and the pellet was resuspended in a suitable volume of medium containing 2.5 nM PEA or DMSO.

Effects of PEA on Hyperactivated Sperm Motility—The effects of PEA on sperm capacitation were evaluated on the same samples during a 4-hour incubation, at 37°C, in a CO_2 incubator (5% CO_2 in air at 95% relative humidity).

Each sample contained 10×10^6 cells/mL. Aliquots (3 μL) of both sperm samples were withdrawn during the incubation at various times; these aliquots were placed in a MicroCell chamber (Conception Technologies, San Diego, Calif) and assayed using CASA (CellTrak VP110; Motion Analysis). The kinematic values for at least 200 motile spermatozoa were analyzed in each sample and were considered hyperactivated in cells with VCL values equal to 100 μm/s or greater, amplitude of lateral head displacement (ALH) values equal to 5 μm or greater, and linearity (LIN) values of less than 60% (Mortimer and Mortimer, 1990). HA is linked to the process of capacitation (Katz et al, 1989) and has been proposed as one of the criteria for evaluating the fertilizing potential of human spermatozoa (Chan et al, 1992; Johnston et al, 1994; Suarez, 1996; Kay and Robertson, 1998). As a clinical application, human sperm HA has been shown to be directly correlated with the success of in vitro fertilization (Wang et al, 1993).

Fluorescence Assays: Measurement of Ca^{2+} Influx—For the measurement of Ca^{2+} influx, capacitated spermatozoa (10×10^6 cells/mL) were incubated at 37°C in the dark for 45 minutes with the acetoxymethyl ester derivative of the Ca^{2+} chelator fura 2 at a 2-μM final concentration (1 μL/mL of a 2-mM stock solution in DMSO) (Silvestroni et al, 1997). To remove the free fura 2, the cells were then washed twice by centrifugation (500 × *g* for 2 minutes) with the EBSS medium without phenol red and resuspended in the same medium (final concentration = 10×10^6 cells/mL). Each sample was divided into 2 identical aliquots and transferred directly into stirred fluorescence cuvettes. All of these procedures were carried out in the dark to prevent sample photobleaching. Fluorescence that was caused by $[Ca^{2+}]_i$ was monitored with an LS 55 fluorometer (Perkin-Elmer, Norwalk, Conn) equipped with a stirrer. The temperature of the cuvettes was maintained at 37°C in a thermostable chamber using a circulating water bath (F3; HAAKE, Karlsruhe, Germany). After equilibration for 10 minutes in the dark, measurements of external Ca^{2+} influx were begun. PEA in DMSO (or DMSO in the control) and 2 mM Mn^{2+} were added to the sperm suspension after 30 and 60 seconds, respectively, from the beginning of the reaction run. Because the final DMSO concentration in the sample was less than 0.5% (vol/vol), the control experiments were performed with the same DMSO volumes. This DMSO concentration had no effect on the Ca^{2+} influx for the duration of the experiment (Blackmore et al, 1990).

Fura 2 fluorescence was excited at 360 nm, and the emission was measured at 505 nm, according to Blackmore at al (1990). The decrease in fura 2 fluorescence reflects Mn^{2+} influx, which in turn reflects Ca^{2+} influx activity (Hallam and Rink, 1985). The final concentration of DMSO in the sperm suspensions never exceeded 0.2% (vol/vol). Experiments were repeated 6 times using sperm from 6 different donors.

Statistical Significance—Results are expressed as the mean ± SD. The statistical significance of the data was evaluated by a Student's *t* test or analysis of variance.

Results

Effects of PEA on Seminal Functional Parameters and HA

Spermatozoa were incubated both in the presence and absence of 2.5 nM PEA. To evaluate the sensitivity of sper-

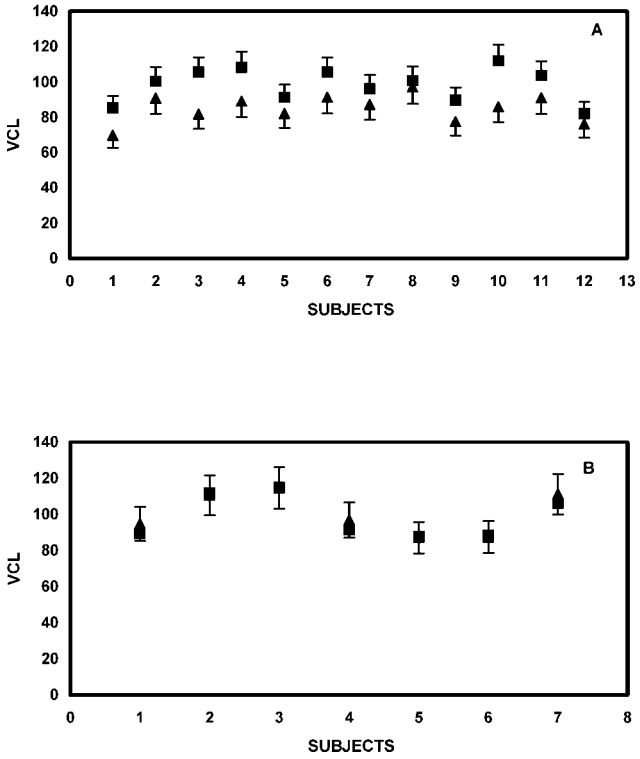

Figure 1. Mean values of curvilinear velocity (VCL) measured, during a 4-hour incubation, in sperm samples from idiopathic infertile men in the absence (▲) and in the presence (■) of 2.5 nM palmitoylethanolamide (PEA). **(Panel A)** Shows samples (12 subjects) that are sensitive to PEA incubation. **(Panel B)** Shows samples (7 subjects) that are insensitive to PEA. **(Panel A)** Statistical significance for VCL was $P < .001$, as evaluated by analysis of variance. **(Panel B)** No statistical significance was evident for VCL.

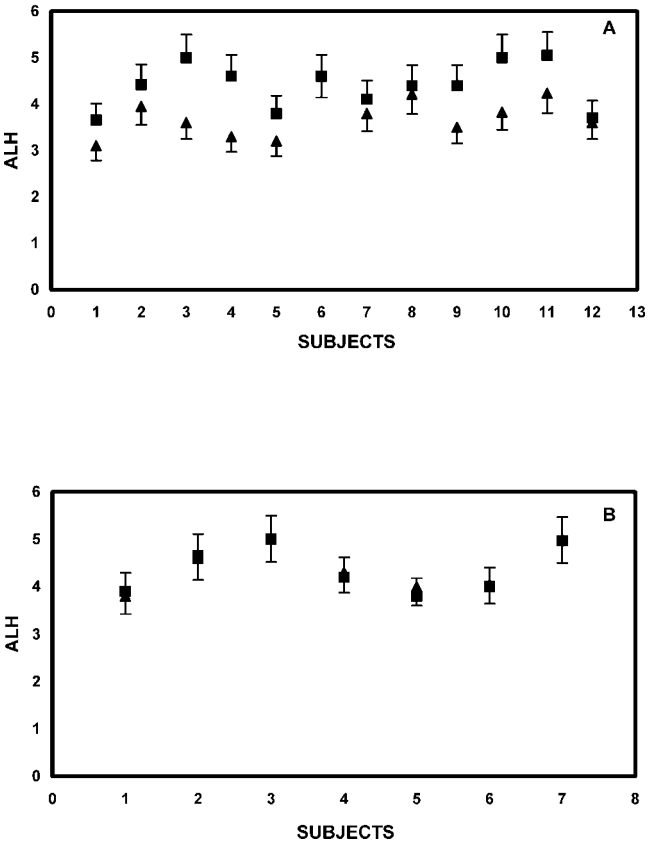

Figure 2. Mean values of amplitude of lateral head displacement (ALH) measured, during a 4-hour incubation, in sperm samples from idiopathic infertile men in the absence (▲) and in the presence (■) of 2.5 nM palmitoylethanolamide (PEA). **(Panel A)** Shows samples (12 subjects) that are sensitive to PEA incubation. **(Panel B)** Shows samples (7 subjects) that are insensitive to PEA. **(Panel A)** Statistical significance for ALH was $P < .01$, as evaluated by analysis of variance. **(Panel B)** No statistical significance was evident for ALH.

matozoa to incubation with PEA, we focused our attention on the values of VCL, ALH, and LIN, because their modification is an important index for the evaluation of sperm capacitation and HA (Mortimer and Mortimer, 1990). Figures 1 and 2 show the effect of PEA on VCL and ALH, respectively, measured during a 4-hour incubation on each sample. LIN is not shown because no PEA-induced changes were evident in the samples for this parameter. We have identified 12 samples (63% of the total), which present a PEA-induced increase in both parameters, with respect to the control (Figures 1 and 2, panel A). The other 7 samples (37% of the total) do not show a sensitivity to PEA (Figures 1 and 2, panel B). On the basis of these results, we have identified 2 groups of samples (and thus patients): 1) samples that are sensitive to PEA (group I, 12 patients), and 2) samples that are insensitive to PEA (group II, 7 patients). All kinematic parameters were evaluated in the 2 groups and compared.

Table 1 shows that, before capacitation, the mean values of sperm cell concentration, the percentages of total motility (a + b + c), and the VSL, VCL, and ALH values

Table 1. *Kinematic parameters measured on spermatozoa from group I (12 samples) and group II (7 samples) before the capacitation; data are presented as the mean ± SD of values**

	ALH	Mil/mL	Motility, %	VSL	VCL	LIN
Group I	36 ± 14†	36 ± 12‡	19 ± 2†	45 ± 11†	47 ± 10	2.75 ± 0.5†
Group II	58 ± 18	62 ± 6	22 ± 2	61 ± 12	40 ± 5	3.50 ± 0.6

* Mil/mL indicates sperm cell concentration; Motility, %, percent motility (a + b + c); VSL, straight-line velocity; VCL, curvilinear velocity; LIN, linearity; and ALH, amplitude of lateral head displacement.

† $P < .01$.

‡ $P < .001$ significance, group I vs group II.

Table 2. *Kinematic parameters measured on spermatozoa from group I (12 samples) and group II (7 samples) during capacitation performed in the absence and in the presence of 2.5 nM PEA; data are presented as the mean ± SD of all values measured during a 4-hour incubation**

	Motility, %	VSL	VCL	LIN	ALH
Group I					
No PEA	64 ± 15§	27 ± 4§	86 ± 7‖	34 ± 6	3.81 ± 0.4‖
With PEA	71 ± 15	29 ± 4	100 ± 8‡	32 ± 6	4.50 ± 0.5†
Group II					
No PEA	79 ± 9	31 ± 4	100 ± 11	33 ± 3	4.50 ± 0.5
With PEA	79 ± 9	29 ± 4	99 ± 11	32 ± 3	4.40 ± 0.6

* Motility, % indicates percent motility (a + b + c); VSL, straight-line velocity; VCL, curvilinear velocity; LIN, linearity; ALH, amplitude of lateral head displacement; and PEA, palmitoylethanolamide.
† $P < .01$.
‡ $P < .001$ significance, group I in the presence and absence of PEA.
§ $P < .05$.
‖ $P < .01$ significance, group I vs group II in the absence of PEA.

for group I are significantly lower than the same parameters measured for group II. No significant differences between the 2 groups were evident in LIN (Table 1).

The effects of the capacitation process on the values of the percentage of motility, VSL, VCL, ALH, and LIN measured for groups I and II are shown in Table 2. Values are presented as the average of the mean values measured for the samples of each group during a 4-hour incubation and performed either in the absence or presence of 2.5 nM PEA. In group I, PEA induced a significant increase in VCL and ALH ($P < .001$ and $P < .01$, respectively). PEA did not significantly modify seminal parameters in group II samples, compared with their controls (Table 2). It is noteworthy that, when spermatozoa were capacitated for 4 hours at 37°C in the absence of 2.5 nM PEA, the mean percentage of motility was significantly different between groups I and II (Table 2). In the presence of PEA, a slight increase in motility was evident in group I (from 64% ± 15% in control samples to 71.0% ± 15% in treated samples) (Table 2). This parameter remained the same in group II: 79% ± 9% in control samples and 79% ± 11% in treated samples (Table 2). In the presence of PEA, not significantly different values for motility were obtained when intragroup comparisons were made for the 2 groups (Table 2), even though the motility remained higher for group II when intergroup comparisons were made for the 2 groups. Moreover, group I, after treatment with PEA, and group II had similar VSL, VCL, LIN, and ALH values (Table 2).

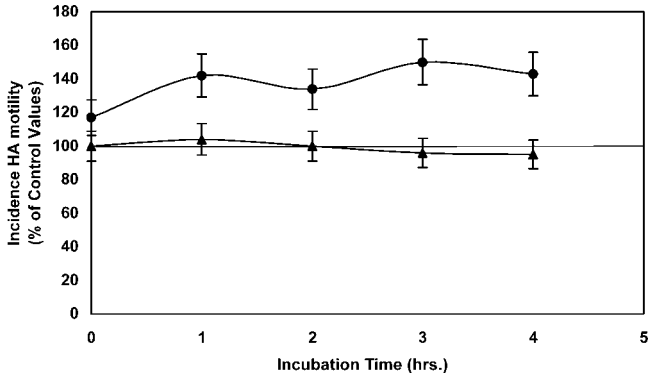

Figure 3. Effects of 2.5 nM palmitoylethanolamide (PEA) on hyperactivated motility (HA) in sperm during incubation for 4 hours. Incidence of HA in sperm treated with 2.5 nM PEA: (●) group I and (▲) group II. Data are expressed as a percentage of control values. In group I (12 samples), PEA induced a significant increase in the percentage of HA at each time point tested ($P < .01$ at 0 hours; $P < .001$ at 1, 2, 3, and 4 hours; *t* test). In group II (7 samples), PEA induced no significant changes in HA during all times of experiments. The effects of PEA on HA in group I (12 samples) were significantly different from those produced in group II (7 samples) during all times of experiments ($P < .001$; *t* test).

Figure 3 shows the effects of PEA on HA measured for both groups at specific time points (0, 1, 2, 3, and 4 hours). Data are expressed as a percentage of the control values. Measurements for HA were begun at the end of performing the gradient procedure (time 0): the HA incidence at this time in the controls of group II was 14% ± 4%, while no HA was measured in group I controls. For each time point, the statistical significance was calculated by determining, for each group, the mean of the incidence of HA in the presence of PEA with respect to its incidence in the presence of the control DMSO sample.

In group I, PEA induced a significant increase in the percentage of HA at each time point tested ($P < .01$ at 0 hours of incubation, and $P < .001$ at 1, 2, 3, and 4 hours of incubation), whereas, in group II, PEA induced no significant changes in the percentage of HA with respect to the controls for any time point during the experiments.

To compare the response to PEA incubation of idiopathic infertile patients and fertile subjects, we performed the same experiments on sperm from men of proven fertility. These samples show kinematic parameters (concentration, percentage of motility, VSL, VCL, LIN, and ALH) similar to those of group II both before and after capacitation (data not shown). Moreover, in samples of

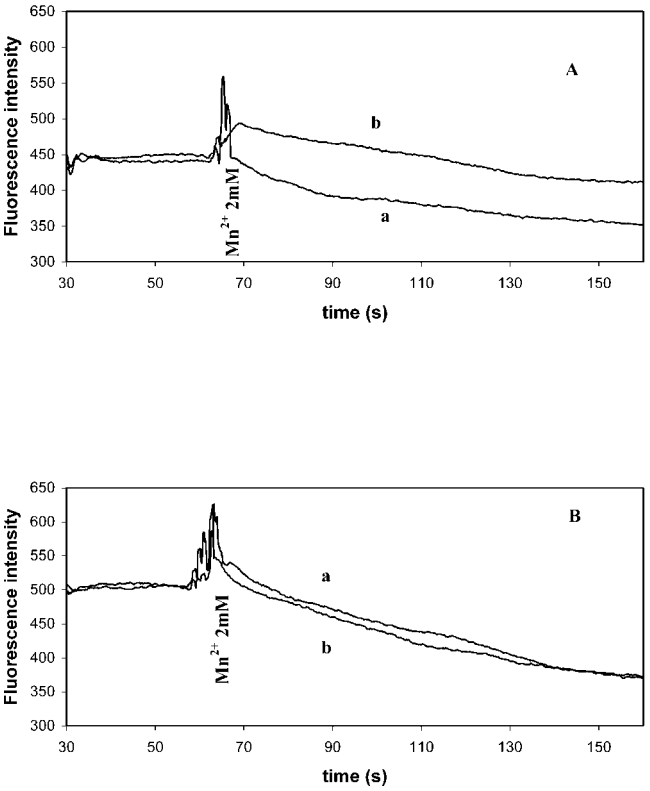

Figure 4. Effect of 2.5 nM palmitoylethanolamide (PEA) on the induction of Ca^{2+} influx measured by observing the ability of Mn^{2+} added extracellularly to quench fura 2. For sperm loaded with fura 2, either 2.5 nM PEA (curve a) or DMSO (curve b) was added at 30 seconds, and finally, 2.0 mM Mn^{2+} was added at 60 seconds to all samples in this experiment. The decrease in fura 2 fluorescence (excited at 360 nm) reflects Mn^{2+} influx, which in turn reflects Ca^{2+} influx activity. N = 6 experiments using sperm from 6 different donors. Group I **(Panel A)** includes 4 samples, and group II **(Panel B)** includes 2 samples.

fertile men, PEA does not significantly modify the kinematic parameters and HA (data not shown).

Effects of PEA on Ca^{2+} Influx

The measure of the ability of Mn^{2+} to quench intracellular fura 2 has been used as a sensitive approach to measure Ca^{2+} influx in sperm (Hallam and Rink, 1985; Blackmore et al, 1990, 1991). In fact, the divalent cation Mn^{2+} is carried by Ca^{2+} channels; hence, its influx into cells is a measure of Ca^{2+} influx activity (Hallam and Rink, 1985). Figure 4 shows that 2 mM Mn^{2+}, when added to fura 2–loaded sperm, produced a gradual decrease in fura 2 fluorescence in the controls of groups I and II (curve b of panels A and B, respectively). This fura 2 fluorescence decrease is greatly accentuated when 2.5 nM PEA is added to group I samples (Figure 4, panel A, curve a). On the contrary, samples classified as group II (Figure 4, panel B, curve a) and samples from fertile patients (data not shown) are almost insensitive to PEA, which indicates that, in these cells, this molecule has no effect on Ca^{2+} influx. That PEA induced a large decrease in fura 2 fluorescence in group I samples could suggest that, in this group, the effect of PEA on sperm hyperactivation involves an influx of Ca^{2+} through the plasma membrane Ca^{2+} channel.

Discussion

Defective sperm function is the most common cause of infertility, but it is difficult to evaluate and treat. It is known that some physical and functional characteristics of the sperm plasma membrane are largely involved in sperm fertilization capacity (Langlais et al, 1988; Hoshi et al, 1990). Our previous studies of men affected by idiopathic infertility demonstrated that spermatozoa from oligozoospermic men and some normozoospermic men (as defined by World Health Organization criteria) are characterized by plasma membrane alterations (decreased membrane polarity) (Ambrosini et al, 2001), which are likely related to altered in vitro–induced capacitation (Ambrosini et al, 2003). We suggested that these membrane alterations are physiologically relevant for in vivo capacitation and that they are a possible cause of infertility in these subjects (Ambrosini et al, 2003).

Moreover, we demonstrated that PEA increases the rate of in vitro capacitation in sperm cells characterized with plasma membrane alterations (decreased membrane polarity) (Ambrosini et al, 2003). It has been proposed that this PEA effect is related to the bilayer lipid structural features of these sperm plasma membranes (Ambrosini et al, 2003). As part of the complex process of capacitation, spermatozoa develop HA (Kay and Robertson, 1998). The acquisition of HA seems to render spermatozoa capable of generating the ''power'' needed for penetrating the extracellular matrix of the egg; fertilization is not possible in the absence of this highly active form of motility (Quill et al, 2003).

Previous studies have demonstrated that semen from idiopathic infertile men shows much lower hyperactivation than semen from fertile subjects (Peedicayil et al, 1997). Spermatozoa from idiopathic infertile men have been categorized into 2 groups: one with a lower hyperactivation rate and one with a higher hyperactivation rate (Peedicayil et al, 1997). No significant differences were observed in the rate of the acrosome reaction between idiopathic infertile men and fertile men (Peedicayil et al, 1997).

In this work on idiopathic infertile men, we have identified 2 groups of sperm samples: 1) those with kinematic parameters sensitive to PEA (group I), and 2) those with kinematic parameters insensitive to PEA (group II).

In the absence of PEA, the behavior of these samples is in line with that observed for the groups identified by Peedicayil et al (1997), because group II shows a quite

relevant HA, while group I does not show hyperactivation. The incubation of group I samples with 2.5 nM PEA modifies the kinematic parameters of these sperm cells so that, during a 4-hour incubation, no more significant differences in these parameters were found between groups I and II.

Many physiological factors are essential for the initiation and maintenance of HA in vitro. It has been demonstrated that Ca^{2+} plays a major role in regulating HA, and the involvement of plasma membrane Ca^{2+} channels is thought to play a role in initiating and maintaining hyperactivation (Quill et al, 2003). Even if the mechanism by which intracellular Ca^{2+} concentrations increase remains unknown, a variety of widely expressed calcium-permeable channels (voltage gated, cyclic nucleotide gated, and transient receptor potential) have been identified in sperm cells (Wiesner et al, 1998; Wennemuth et al, 2000; Castellano et al, 2003). Moreover, the involvement of extracellular Ca^{2+} in the regulation of HA was supported by the recent discovery of a family of sperm-specific cation channels, located principally on the sperm flagellum, where they may act as regulators of sperm motility, because the activation of these proteins drives a hyperactivated form of motility that is essential for fertilization (Quill et al, 2003). Data obtained in our work, using fluorescence, show that, in samples classified as group I, PEA induces an increase in Ca^{2+} influx within the sperm cell, whereas in group II, this behavior is not evident (Figure 4). These data suggest that the PEA modulation of HA in sperm involves plasma membrane Ca^{2+} channels.

Although the mechanism of the PEA effect on these proteins cannot be easily clarified, some hypotheses can be attempted.

A previous work (Schuel et al, 2002b) hypothesized that R-methanandamide (AM-356), a potent and metabolically stable AEA analog, modulates HA by regulating Ca^{2+} channels, likely through G-protein–associated cannabinoid receptors. Although PEA is not thought to be a ligand of the cannabinoid receptor CB1, which is expressed in spermatozoa (Schuel et al, 2002b), the possible interaction with other cannabinoid receptors, which could be contained in human sperm (Schuel et al, 2002b), cannot be excluded a priori. Moreover, the action of PEA on other possible receptors that modulate the Ca^{2+} influx in human sperm is possible. In other tissues, such as human embryonic kidney cells (Smart et al, 2002; Vandervoorde et al, 2003), PEA produces a Ca^{2+} influx both per se and an augmentation of the cation transport induced by AEA (‘‘entourage effect’’). This effect is observed in cells that express a vanilloid receptor. However, Ca^{2+} influx could be modulated through other mechanisms, which may not be mutually exclusive. For example, the lipid nature of this molecule suggests that its action can be performed, at least partially, through physical interactions with the lipid part of the cellular membrane. The possible preferential localization of PEA in specific domains, with different lipid composition, could give rise to microenvironments with different structural and physicochemical features (Ambrosini et al, 1993; Zolese et al, 2003), which could modify directly and/or indirectly calcium channel activity. This hypothesis could be strengthened in light of our previous results (Ambrosini et al, 2003), which demonstrated that PEA affects capacitation in spermatozoa with altered physicochemical characteristics of the lipid bilayer.

The PEA-induced changes in membrane physicochemical properties could have an important effect on the molecular mechanisms involved in sperm HA, because they could induce an increased opening of calcium channels; however, they could also likely affect membrane permeability to other molecules, which are important for HA (eg, bicarbonate).

The explanation of the molecular mechanism involved in the PEA-induced modulation of HA requires additional research. However, data presented in this work suggest that PEA has a selective effect or action on sperm cells from idiopathic infertile men with low kinematic parameters.

Perhaps these results could be explained by examining what effects modified levels of these compounds would have on individuals in various circumstances; for these reasons, our next work will concern itself with a study of the levels of NEAs in idiopathic infertile men.

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