Characterization of the Endocannabinoid System in Human Spermatozoa and Involvement of Transient Receptor Potential Vanilloid 1 Receptor in Their Fertilizing Ability

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Human spermatozoa express type-1 cannabinoid receptor (CB1), whose activation by anandamide (AEA) affects motility and acrosome reaction (AR). In this study, we extended the characterization of the AEA-related endocannabinoid system in human spermatozoa, and we focused on the involvement of the AEA-binding vanilloid receptor (TRPV1) in their fertilizing ability. Protein expression was revealed for CB1 (~56 kDa), TRPV1 (~95 kDa), AEA-synthesizing phospholipase D (NAPE-PLD) (~46 kDa), and AEA-hydrolyzing enzyme [fatty acid amide hydrolase (FAAH), ~66 kDa]. Both AEA-binding receptors (CB1 and TRPV1) exhibited a functional binding activity; enzymatic activity was demonstrated for NAPE-PLD, FAAH, and the purported endocannabinoid membrane transporter (EMT). Immunoreactivity for CB1, NAPE-PLD, and FAAH was localized in the postacrosomal region and in the midpiece, whereas for TRPV1, it was restricted to the postacrosomal region. Capsazepine (CPZ), a selective antagonist of TRPV1, inhibited progesterone (P)-enhanced sperm/oocyte fusion, as evaluated by the hamster egg penetration test. This inhibition was due to a reduction of the P-induced AR rate above the spontaneous AR rate, which was instead increased. The sperm exposure to OMDM-1, a specific inhibitor of EMT, prevented the promoting effect of CPZ on spontaneous AR rate and restored the sperm responsiveness to P. No significant effects could be observed on sperm motility. In conclusion, this study provides unprecedented evidence that human spermatozoa exhibit a completely functional endocannabinoid system related to AEA and that the AEA-binding TRPV1 receptor could be involved in the sperm fertilizing ability. (*Endocrinology* 150: 4692–4700, 2009)

Endocannabinoids include two families of bioactive lipids, the fatty acid amides, and the monoacylglycerols. The best characterized prototype members of these two families are *N*-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG), which are both able to act primarily at type-1 (CB1) and type-2 (CB2) cannabinoid receptors (1) as well as at the orphan G protein-coupled receptor GPR55, which has been recently identified as a novel cannabinoid receptor (2, 3). Therefore, they reproduce some of the biological actions of the natural *Cannabis sativa* components (the cannabinoids), of which Δ^9-tetrahydrocannabinol (THC) is the most prominent member (1, 4, 5). It should be recalled that THC has been recognized as the major threat for repro-

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Abbreviations: AEA, Anandamide; 2-AG, 2-arachidonoylglycerol; AR, acrosome reaction; BWW, Biggers, Whitten, and Whittingham; CASA, computer-assisted semen analysis; CB1, type-1 cannabinoid receptor; CPZ, capsazepine; DMSO, dimethylsulfoxide; EMT, endocannabinoid membrane transporter; FAAH, fatty acid amide hydrolase; HEPT, hamster egg penetration test; HRP, horseradish peroxidase; HSA, human serum albumin; NAPE, *N*-acylphosphatidylthanolamine; NAPE-PLD, NAPE-hydrolyzing phospholipase D; P, progesterone; RT, room temperature; RTX, resinferatoxin; THC, Δ^9-tetrahydrocannabinol; TRPV1, transient receptor potential vanilloid 1.

ductive events in marijuana users (6). AEA, but not 2-AG, behaves also as an endovanilloid that binds to the type-1 vanilloid receptor (transient receptor potential vanilloid 1, TRPV1) at an intracellular site (7–9).

TRPV1 is a six *trans*-membrane spanning nonselective cation channel with intracellular N and C termini, that belongs to the family of TRP ion channels, whose expression is largely associated with small-diameter primary afferent fibers and some nociceptor efferent neurons (8, 10, 11). TRPV1 is a molecular sensor of physical stimuli and can be activated also by naturally occurring vanilloids, such as the pungent ingredient of hot chili peppers, capsaicin, and the plant-derived toxin resinferatoxin (RTX) (9). Although initially controversial, it is now firmly established that TRPV1 is expressed in nonneuronal cells, such as keratinocytes and epithelial and endothelial cells, where it could play a wide variety of physiological functions (12).

The biological activity of AEA at its receptors is under a metabolic control, *i.e.* it depends on the endogenous level of this substance within the cell (4). AEA is not stored in vesicles like other cellular mediators but instead is released on demand from membrane phospholipid precursors, the *N*-acylphosphatidylthanolamines (NAPE), through the activity of NAPE-hydrolyzing phospholipase D (NAPE-PLD) (13). Furthermore, a bidirectional transport mechanism across the plasma membrane, possibly catalyzed by a purported endocannabinoid membrane transporter (EMT), is responsible for the uptake of extracellular AEA (14–16). Once inside the cell, AEA is degraded to ethanolamine and arachidonic acid by the endomembrane-bound fatty acid amide hydrolase (FAAH) (17, 18). Together with AEA and congeners, metabolic enzymes and target receptors collectively form the endocannabinoid system (4, 5, 19).

Evidence has been accumulated that exogenous cannabinoids could play a role in the modulation of male reproductive functions (6, 20). Chronic administration of THC to animals induces impotence (21), lowers testosterone secretion, and reduces the production, motility, and viability of spermatozoa (22, 23). More recently, it has been demonstrated that THC *in vitro* inhibits human sperm motility and acrosome reaction (AR), both spontaneous and induced by calcium ionophore A23187 (24). Additionally, the binding of exogenous AEA to a CB receptor expressed on sea urchin spermatozoa is known to inhibit egg jelly-stimulated AR (25–27). Further interesting contributions arose from the gene knockout technology. In a recent study, the percentage of motile spermatozoa recovered from epididymis of CB1 knockout mice was higher with respect to wild-type mice, suggesting a physiological inhibitory control of endocannabinoids on sperm motility via CB1 in the epididymis (28). More recently, the genetic loss of FAAH in the mouse resulted in increased levels of AEA in the reproductive system, leading to an impairment of sperm fertilizing ability, which was rescued by superimposing deletion of CB1 (29). Taken together, these findings suggest that exogenous cannabinoid signaling may affect male fertility. However, as recently demonstrated in the boar (*Suus scropha*), mammalian spermatozoa might also contain endogenous AEA, whose endovanilloid intracellular action on TRPV1 could play a role during capacitation (30). In boar spermatozoa, a fully functional endocannabinoid system that binds (CB1 and TRPV1), synthesizes (NAPE-PLD), and degrades (EMT and FAAH) AEA has been demonstrated (30). More recently, CB receptors and FAAH expression has been reported also in bovine sperm cells (31). In addition, the metabolically stable AEA analog methanandamide was shown to inhibit, via CB1, boar sperm capacitation and, consequently, the physiological AR triggered by the zona pellucida (30). Instead, an increase of intracellular AEA during capacitation has been shown to prevent spontaneous exocytosis, noninstrumental to fertilization, by acting on TRPV1 (30). Therefore, the same substance (AEA) is used to bring different signals to sperm cells, depending on the target receptor (CB1 or TRPV1) that is activated.

On the other hand, little is known about a possible involvement of the endocannabinoid system in human sperm functions. Radioligand binding studies with the synthetic cannabinoid [^{3}H]CP55.940 provided the first evidence for cannabinoid receptors in human spermatozoa (32), and AEA has been detected in fluids of male and female reproductive tracts (32, 33). More recently, human spermatozoa have been shown to express CB1 (34). Remarkably, activation of the latter receptor by exogenous AEA was shown to inhibit motility, mitochondrial activity, and AR during capacitation (34). These findings extend previous data on the inhibitory effect of methanandamide and THC on human sperm hyperactivated motility and zona pellucida binding (32, 33). These data are in keeping with those produced in boar sperm. However, the ability of human sperm to bind, synthesize, and degrade AEA has never been reported. In particular, no data are as yet available on the activity and expression of TRPV1 in human sperm, despite the critical role of this receptor demonstrated in boar spermatozoa (30).

The aim of the present study was a biochemical and functional characterization of the AEA-related endocannabinoid system in human sperm, with a focus on the possible implications of TRPV1 in controlling sperm fertilizing ability.

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Materials and Methods

Chemicals

Chemicals were of the purest analytical grade. Anandamide (*N*-arachidonoylethanolamine, AEA), OMDM-1, RTX, and progesterone (P) were purchased from Sigma Chemical Co. (St. Louis, MO). Capsazepine (*N*-[2-(4-chlorophenyl) ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbothioamide, CPZ), and URB597 were from Calbiochem (San Diego, CA). AEA-ethanolamine-1-[^{3}H] (60 Ci/mmol), [^{3}H]CP55.940 (126 Ci/mmol) and [^{3}H]RTX (43 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). *N*-[^{3}H]Arachidonoyl-phosphatidylethanolamine (200 Ci/mmol) was from ARC (St. Louis, MO). SR141716 and SR144528 were kind gifts of Sanofi-Aventis Recherche (Montpellier, France). d_8-AEA, rabbit anti-NAPE-PLD, and anti-CB1 polyclonal antibodies were from Cayman Chemicals (Ann Arbor, MI). Commercial anti-NAPE-PLD antibody recognized the amino acids sequence 378–390 of human NAPE-PLD, whereas anti-CB1 antibody was raised against the amino acids sequence 1–14 of human CB1. Rabbit anti-CB2 polyclonal antibody, directed against CB2 amino acids 1–33, was from ABR-Affinity BioReagents (Golden, CO). Rabbit anti-TRPV1 polyclonal antibody, recognizing human TRPV1 amino acids 1–150, and anti-FAAH polyclonal antibody, raised against the FAAH amino acids 378–390, was from Santa Cruz Biotechnologies (Santa Cruz, CA). Biotin-conjugated mouse antirabbit secondary antibody and Cy3-streptavidin were from Sigma. Ig fraction of preimmune rabbit serum and the EnVision + Dual Link System-Horseradish Peroxidase (HRP) kit were purchased from DakoCytomation (Carpinteria, CA). P, CPZ, SR141716, and OMDM-1 were prepared daily as stock solutions in dimethylsulfoxide (DMSO) and were diluted in Biggers, Whitten, and Whittingham (BWW) medium to obtain the working concentrations just before use.

Semen samples and sperm processing

For immunocytochemical and functional tests, semen samples were collected from 20 normozoospermic postgraduate medical students of proven fertility. For biochemical assays, semen samples were obtained from 55 normozoospermic men who sought medical care for infertile marriage.

The study was approved by the local Institutional Review Board, and all subjects signed an informed consent statement.

All semen samples were collected by masturbation, according to the World Health Organization (WHO)-recommended procedure (35). All samples were produced into sterile containers and left for at least 30 min to liquefy before processing. Motile sperm suspensions were obtained by swim up procedure. Briefly, spermatozoa were washed twice at $700 \times g$ for 7 min in BWW medium containing 0.1% human serum albumin (HSA, fraction V, no. 1653). After the second centrifugation, supernatants were removed by aspiration, leaving 0.5 ml on the pellet, and after 30 min of incubation, supernatants containing highly concentrated motile sperm were carefully aspirated. Motile sperm suspensions used for biochemical assays were washed in PBS by centrifugation ($700 \times g$ for 10 min), and pellets were resuspended in 1 ml of the same buffer, frozen, and stored at −80 C until analyses.

Capacitated suspensions used for sperm function tests were obtained by incubation of motile spermatozoa (7×10^6/ml) in BWW with the addition of 1% HSA at 37 C in an atmosphere of 5% CO_2/95% air for 5 h.

Endogenous levels of AEA

Purified sperm were subjected to lipid extraction with chloroform/methanol (2:1, vol/vol), in the presence of d_8-AEA as internal standard (36). The organic phase was dried and then analyzed by liquid chromatography-electrospray ionization mass spectrometry, using a single quadrupole API-150 EX mass spectrometer (Applied Biosystems, Foster City, CA) in conjunction with a PerkinElmer liquid chromatography system. Quantitative analysis was performed by selected ion recording over the respective sodiated molecular ions (36).

Receptor binding assays

Cannabinoid and vanilloid receptor studies were performed by rapid filtration assays, using the synthetic cannabinoid [^{3}H]CP55.940 (500 pM) and the TRPV1 agonist [^{3}H]RTX (500 pM), respectively (37, 38). In all experiments, nonspecific binding was determined in the presence of cold agonists (1 μM CP55.940 or 1 μM RTX) and was further corroborated by selective antagonists [0.1 μM SR141716 for CB1 (39) and 1 μM CPZ for TRPV1 (40)], as reported (30). Also the effect of 0.1 μM SR144528, a selective CB2 antagonist (41), was ascertained under the same experimental conditions.

Metabolism of AEA

The synthesis of [^{3}H]AEA through the activity of NAPE-PLD (E.C. 3.1.4.4) was assayed in sperm homogenates (100 μg/test) by using 100 μM *N*-[^{3}H]arachidonoyl-phosphatidylethanolamine and reversed-phase HPLC, as reported (42). The hydrolysis of 10 μM [^{3}H]AEA by FAAH (E.C. 3.5.1.4) was assayed in sperm extracts (50 μg/test), by measuring the release of [^{3}H]ethanolamine as reported (43). The uptake of AEA was measured on intact sperm (10×10^6 cells per test), incubated with 400 nM [^{3}H]AEA for 15 min at 37 C (14). The effect of the FAAH inhibitor URB597 (43) or of the EMT inhibitor OMDM-1 (44) was determined by adding each substance directly to the incubation medium (45). On the other hand, it was not possible to extend this analysis to NAPE-PLD, because selective inhibitors are not yet available for this enzyme.

Expression of NAPE-PLD, FAAH, CB1, CB2, and TRPV1

Sperm homogenates (50 μg/lane) were subjected to SDS-PAGE on a 10% polyacrylamide gel and electroblotted onto a polyvinylidene difluoride membrane. Blots were blocked with 10% nonfat dry milk and 5% BSA for 2 h and then incubated with anti-NAPE-PLD (diluted 1:1000), anti-FAAH (diluted 1:1000), anti-CB1 (diluted 1:100), anti-CB2 (diluted 1:100), or anti-TRPV1 (diluted 1:200) primary antibodies. After washing and incubation with the HRP-conjugated secondary antibody (1:1000), detection was carried out using West Dura Chemiluminescence System (Pierce, Rockford, IL). As positive controls, homogenates of mouse brain (for NAPE-PLD, FAAH, and CB1), mouse spleen (for CB2), or HeLa cells (for TRPV1) were used under the same experimental conditions.

Immunocytochemistry

Localization of TRPV1, FAAH, and NAPE-PLD antigens on human spermatozoa was investigated by immunoperoxidase staining experiments. Because TRPV1 and FAAH are intracellular proteins not accessible to antibodies in intact cells (30, 46),

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motile spermatozoa were smeared, fixed in methanol, and permeabilized with 0.1% (vol/vol) Triton X-100 in PBS (pH 7.4) plus 0.1% HSA for 30 min at room temperature (RT). The immunostaining procedure was performed according to the protocol of the Envision + Dual Link System-HRP kit (DakoCytomation). The high sensitivity of this system is based on an HRP-labeled polymer that is conjugated with secondary antibodies. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide containing sodium azide. Rabbit anti-TRPV1 (1:10), anti-FAAH (1:10), or anti-NAPE-PLD (1:10) antibodies were applied, and slides were left in humidity chambers at RT for 2 h. After washing in PBS for 5 min, samples were incubated with peroxidase-labeled polymer conjugated to goat antirabbit $F(ab')_2$ Ig for 1 h at RT, followed by a washing step in PBS. Immunocytochemical reactions were revealed using 3′,3′-diaminobenzidine, included in the EnVision kit, as a chromogen. Slides were dehydrated, coverslipped with permanent mounting media, and assessed under light microscopy (Leica DMLB, Wetzlar, Germany). Immunolocalization of CB1 was investigated with immunofluorescence on unfixed spermatozoa. Briefly, motile sperm suspensions were incubated with a rabbit anti-CB1 antibody (1:10) at RT for 1 h. After two centrifugations, specific labeling was detected via a biotin-conjugated mouse antirabbit secondary antibody (1:100) and Cy3-streptavidin (1:100). After repeated washing, spermatozoa were smeared, mounted in PBS-glycerol, and observed under a fluorescence microscope (Leica). The immunostaining specificity was always checked by running negative controls with the replacement of specific primary antibodies with Ig fractions from nonimmunized rabbits at the same concentration.

Sperm function tests

The possible role of TRPV1 in sperm functions involved in fertilization was explored by evaluating the effect of CPZ, a selective antagonist of TRPV1 (47), on sperm/oocyte fusion and on AR.

The effect of CPZ on the sperm/oocyte fusion was evaluated by means of the P-enhanced hamster egg penetration test (HEPT), performed as previously described (48). Briefly, motile sperm suspensions were capacitated in the presence of 1 μM CPZ or a proper dilution of DMSO as control. After 5 h of capacitation, spermatozoa were exposed to 5 μM P for 15 min before incubation with oocytes. Spermatozoa were washed before the incubation with oocytes to exclude a possible direct effect of reagents on oocytes. Standard procedures were used for the recruitment of hamster oocytes (35). Ten to 15 zona-free oocytes were added to droplets of 100 μl, each containing 7×10^5 motile spermatozoa. After 3 h coincubation at 37 C in an atmosphere of 5% CO_2/95% air, oocytes were recovered from the droplets, washed free of loosely adherent spermatozoa, and colored with SYBR14 (1:500). The latter compound is a nuclear membrane-permeant fluorochrome, specific for DNA. It stains nuclei of living spermatozoa fluorescing bright green. Ova were examined for evidence of swollen sperm heads, as the criterion of sperm penetration, under a microscope equipped with epifluorescence (Leica). The number of spermatozoa penetrating each egg was assessed and expressed as penetration index, *i.e.* total number of penetrations per total number of oocytes.

A set of experiments was carried out to evaluate the effect of CPZ on spontaneous and P-induced ARs. Briefly, motile sperm suspensions were capacitated in the presence of 1 μM CPZ or a proper dilution of DMSO as control. After 5 h of capacitation, sperm suspensions were divided into two aliquots (100 μl), each containing 0.7×10^6 motile spermatozoa; an aliquot was exposed to P (15 μM) and the other one to a proper dilution of DMSO for 30 min. In an additional set of experiments, we also evaluated the effect of the concomitant addition of OMDM-1 (10 μM), an EMT inhibitor (44), on the activity of CPZ. Because AEA transport by EMT across the cell membrane is supposed to be bidirectional (14–16), with this approach, it should be possible to minimize the export of AEA from spermatozoa (45). The increased intracellular concentration of AEA was expected to displace CPZ from TRPV1, thereby preventing its inhibition of the receptor. Finally, because some of the AEA synthesized within the spermatozoa could be exported in the extracellular medium by the bidirectional EMT, the last set of experiments was aimed at ruling out any interference of AEA signaling via CB1 on ARs. Motile sperm suspensions were exposed to SR141716 (0.1 μM), the selective CB1 antagonist, and were capacitated in the absence or in the presence of CPZ (1 μM). AR assessment was performed by processing motile sperm suspensions exposed to the different treatments as described above. Sperm suspensions were centrifuged and then resuspended in hypoosmotic solution for 1 h, according to Aitken *et al.* (49). After centrifugation, spermatozoa were smeared, fixed in methanol, incubated with fluoresceinated *Pisum sativum* agglutinin at 100 μg/ml in PBS for 2 h, washed, and observed under a fluorescent microscope (Leica). At least 200 spermatozoa were counted in each smear, and the percentage of spermatozoa not uniformly fluorescing at the anterior region of the head (reacted spermatozoa) was evaluated. According to Aitken *et al.* (49), only curly-tailed spermatozoa were considered viable and thus scored as true ARs. The true P-induced AR rate was calculated by subtracting the true AR rate observed in samples not exposed to P (spontaneous AR rate) from the true AR rate observed in samples exposed to P.

Sperm motility evaluation with computer-assisted semen analysis

Motility exhibited by sperm suspensions incubated for 5 h in the presence of 1 μM CPZ or DMSO, as control, was evaluated with computer-assisted semen analysis (CASA), using an ATS20 instrument (JCD, Gauville, France). Determinations were performed on sperm suspensions used to evaluate the effect of CPZ on the P-enhanced HEPT before incubation with oocytes in three different settings.

Statistical analysis

Statistical analysis was performed by the SAS statistical software (version 9.1, 2003; SAS Institute, Inc., Cary, NC) and Prism-4 software (GraphPAD Software for Science, San Diego, CA). HEPT results were subjected to the two-way ANOVA to separate replicate from treatment variations (general linear model procedure, PROC GLM). Data from AR assessment were analyzed by Student's paired *t* test or ANOVA, as appropriate. *Post hoc* comparisons between pairs of groups were performed by the Tukey's studentized range, honestly significant difference, test. Biochemical data are expressed as the means ± SD of at least three independent experiments, each performed in duplicate. Functional data are expressed as means ± SEM of at least three independent experiments, each performed in duplicate. Statistical significance was accepted when $P \leq 0.05$.

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TABLE 1. Active elements of the endocannabinoid system in human spermatozoa

Parameter	Control	Inhibitor
Endogenous levels of AEA (pmol/mg protein)	1.11 ± 0.18	
NAPE-PLD activity (pmol/min·mg protein)	189 ± 49	NA
FAAH activity (pmol/min·mg protein)	1489 ± 67	21 ± 3[a]*
EMT activity (pmol/min·mg protein)	0.67 ± 0.04	0.15 ± 0.03[b]*
CB binding (fmol/mg protein)	31 ± 5	10 ± 2[c]*
CB binding (fmol/mg protein)	31 ± 5	30 ± 4[d]
CB binding (fmol/mg protein)	31 ± 5	10 ± 2[e]*
TRPV1 binding (fmol/mg protein)	90.9 ± 1.22	15.5 ± 1.8[f]*

NA, Not available.
[a] Inhibited by 0.1 μM URB597.
[b] Inhibited by 10 μM OMDM-1.
[c] Antagonized by 0.1 μM SR141716.
[d] Antagonized by 0.1 μM SR144528.
[e] Antagonized by a combination of 0.1 μM SR141716 and 0.1 μM SR144528.
[f] Antagonized by 1 μM CPZ.
* $P < 0.05$ *vs.* control.

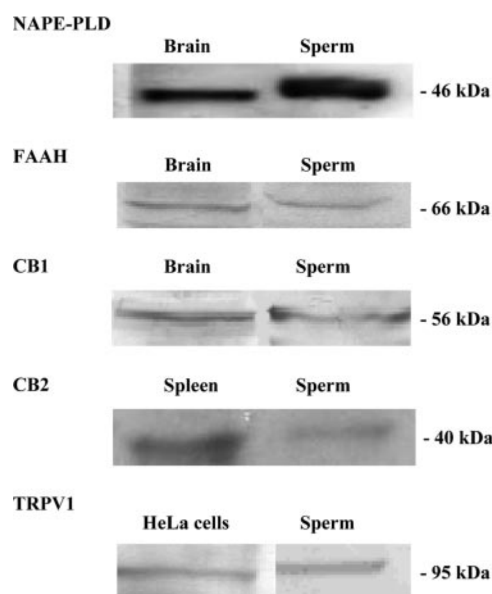

FIG. 1. Western blot analysis of the endocannabinoid system in human sperm. The presence of immunoreactive bands for AEA metabolic enzymes NAPE-PLD and FAAH and for the AEA-binding receptors CB1, CB2, and TRPV1 was ascertained with specific antibodies, able to detect a single band in positive controls (mouse brain, mouse spleen, or HeLa cells). The expected molecular mass of each protein is reported on the *right*.

Results

Endocannabinoid system in human spermatozoa

The activity and expression of the main elements of the AEA-related endocannabinoid system were analyzed in human sperm, and the results are shown in Table 1 and Fig. 1. Much like in boar spermatozoa (30), endogenous AEA was detected in human sperm, which consistently showed active AEA-synthase, NAPE-PLD, and AEA-hydrolase, FAAH (Table 1). Moreover, intact sperm were able to take up AEA through the purported carrier, EMT, as demonstrated by the ability of OMDM-1, a selective EMT inhibitor, to minimize the transport process (Table 1). In addition, human sperm possessed active CB1 and TRPV1, as demonstrated by the binding data and by the effect of selective CB1 or TRPV1 antagonists (Table 1). On the other hand, the lack of effect of the selective CB2 antagonist SR144528 on the binding of [^{3}H]CP55.940 by human sperm suggested the absence of a functional CB2 receptor (Table 1). Also, the observation that the combination of SR141716 and SR144528 had the same effect as SR141716 alone (Table 1) supports the lack of an active CB2 in human sperm.

Further Western blot analysis of human sperm extracts demonstrated the presence of a single immunoreactive band of the expected molecular size of CB1 (~56 kDa), in keeping with previous reports (33, 34). In addition, it showed immunoreactive bands for NAPE-PLD (~46 kDa) and FAAH (~66 kDa) as well as TRPV1 (~95 kDa) that were not yet reported in human sperm (Fig. 1).

Consistent with recent data published by Gervasi *et al.* (31), only a faint band was detected for CB2 (Fig. 1).

Localization of TRPV1, FAAH, NAPE-PLD, and CB1

Immunoreactivity for TRPV1 was restricted to the postacrosomal region of the sperm head (Fig. 2A), whereas it was localized in the postacrosomal region and in the midpiece for FAAH (Fig. 2B), NAPE-PLD (Fig. 2C), and CB1 (Fig. 2F). No labeling was detected in controls exposed to nonimmunized rabbit serum (Fig. 2D).

Effects of CPZ on P-enhanced sperm-oocyte fusion

As shown by Fig. 3, the short exposure of capacitated human spermatozoa to P exerted an expected stimulatory effect on the sperm-oocyte fusion (penetration index = 3.2 ± 0.6 *vs.* 0.7 ± 0.2; $P < 0.05$). The exposure of sperm suspensions to CPZ (1 μM) from the beginning of capacitation led to a significant inhibition of the sperm ability to fuse with oocytes in response to P (1.6 ± 0.4; $P < 0.05$). No change in the percentage of motile spermatozoa, as well as in the quality of motility evaluated with CASA, was observed with any treatment (Fig. 3).

Effect of CPZ on spontaneous and P-induced AR

The exposure of spermatozoa to CPZ (1 μM) during 5 h capacitation produced a significantly higher incidence of spontaneous AR with respect to controls (20.5 ± 1.4 *vs.* 9.2 ± 0.3%, $P = 0.0002$; Fig. 4A).

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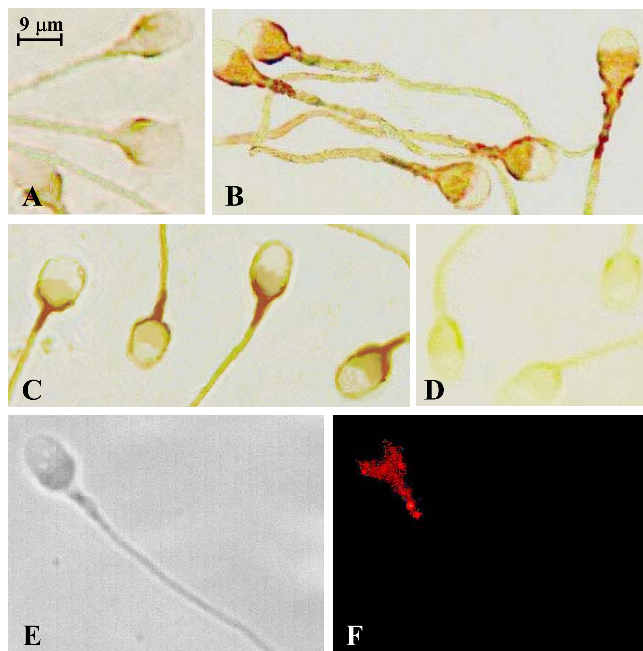

FIG. 2. Immunoreactivity for rabbit anti-TRPV1 (A), FAAH (B), and NAPE-PLD (C) on fixed and permeabilized sperm smears. No immunoperoxidase labeling was detected by replacing specific primary antibodies with Ig fractions from nonimmunized rabbit serum at the same concentration (D). Phase contrast (E) and corresponding immunofluorescence photographs (F) of human spermatozoa incubated with rabbit anti-CB1. *Scale bar*, 9 μm (A–F).

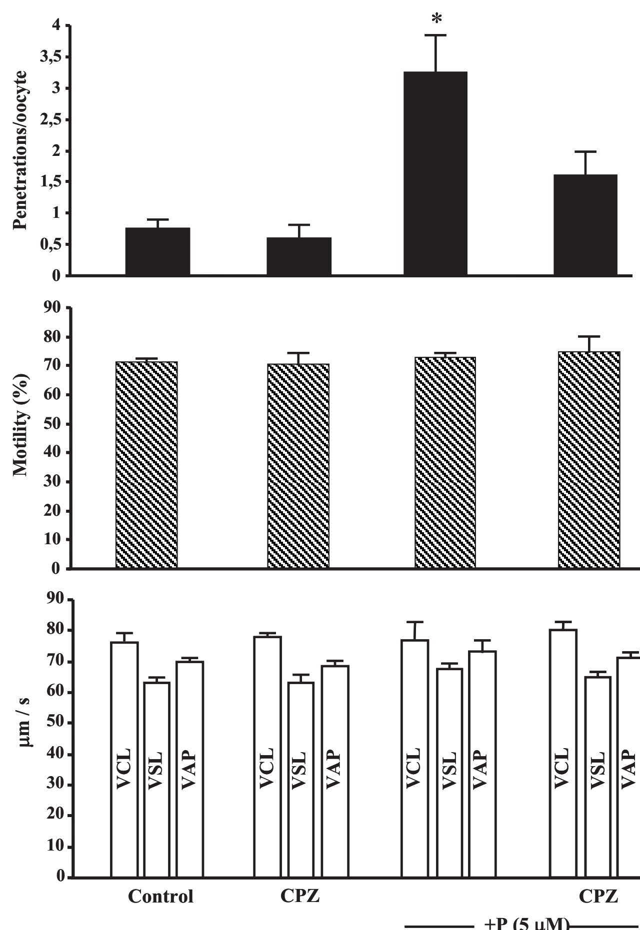

FIG. 3. Effect of the exposure to 1 μM CPZ on the spontaneous and P-stimulated sperm/oocyte fusion (*top*). Overall significance for treatment variation: $P < 0.0001$ (PROC GLM). *, $P < 0.05$ *vs.* all the others (Tukey's test). Number of observations (oocytes) = 389. Number of replicates = 6, with different donors. No change in the percentage of motile spermatozoa (*middle*) or in the quality of motility (*bottom*), evaluated with CASA, was observed with any treatment. VAP, Average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity.

When the AR rate induced by P was evaluated, the increase above the spontaneous AR rate was found to be significantly lower in spermatozoa exposed to 1 μM CPZ (5.3 ± 0.8%) than in controls (15.1 ± 1.6%, $P = 0.0004$; Fig. 4A).

The exposure of motile sperm suspensions to OMDM-1 (10 μM), the specific inhibitor of EMT, from the beginning of the capacitation period (5 h) prevented the stimulatory effect of CPZ on spontaneous AR rate (10.7 ± 0.6 *vs.* 21.3 ± 1.9%, $P < 0.05$) and restored the sperm responsiveness to P (14.6 ± 2.6 *vs.* 1.9 ± 1%, $P < 0.05$; Fig. 4B).

The exposure of spermatozoa to 0.1 μM SR141716, the selective CB1 antagonist, did not affect either spontaneous (6.4 ± 0.6 *vs.* 7.2 ± 0.9%, $P > 0.05$) or CPZ-induced ARs (18.6 ± 1.2 *vs.* 21.3 ± 2.2%, $P > 0.05$; Fig. 5).

Discussion

This study provides unprecedented evidence that human spermatozoa exhibit a completely functional endocannabinoid system related to AEA and that the AEA-binding TRPV1 receptor could play a role in the acquisition of sperm fertilizing ability.

Previous study had shown that human spermatozoa express the CB1 (34), whose activation by extracellular cannabinoids affects motility and ARs (24, 34). An inhibition of AR has been also reported in sea urchin and boar spermatozoa (25, 26, 30). In this study, we demonstrate that human spermatozoa, much like boar spermatozoa (30), besides expressing CB1 that binds extracellular AEA, also exhibit all the other components of the endocannabinoid system needed to synthesize (NAPE-PLD) and degrade (EMT and FAAH) AEA. In addition, they express TRPV1 receptors that are activated by intracellular AEA. All these components are functioning, as demonstrated by the binding activity of both receptors (CB1 and TRPV1) as well as by the enzymatic activity of NAPE-PLD, FAAH, and the transport activity of EMT. It is interesting to recall that a similar endogenous autocrine/paracrine signal system has been demonstrated in sea urchin and mammalian sperm. In fact, they both possess a cholinergic signal system, involving both muscarinic and nicotinic receptors, which regulates several fertilizing functions (50).

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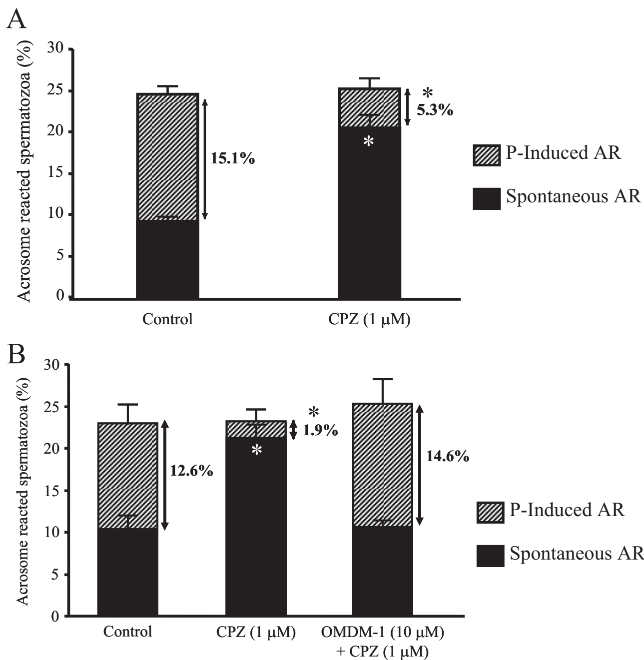

FIG. 4 A, Effect of CPZ on AR. Spontaneous and P-induced AR in spermatozoa capacitated for 5 h in the absence (control) or in the presence of 1 μM CPZ. Spontaneous AR rate was subtracted from total AR rate after exposure to P, to obtain the P-induced AR rate. Number of replicates = 9, with different donors. *, $P = 0.00025$ for CPZ *vs.* control (*t* test). B, Effect of OMDM-1, a specific inhibitor of EMT, on ARs in spermatozoa capacitated for 5 h in the presence of 1 μM CPZ. Number of replicates = 4, with different donors. Overall significance for treatment variation: $P < 0.0001$ with ANOVA. *, $P < 0.05$ for CPZ *vs.* control and *vs.* OMDM-1 + CPZ (Tukey's test).

Cytochemical localization of CB1 and FAAH is superimposable on that reported in boar (30). On the other hand, CB1 was localized also in the acrosomal region of fixed human sperm smears (34), which is at variance with the localization shown here on viable sperm suspensions. Immunoreactivity for TRPV1 was restricted to the postacrosomal region of the human sperm head, whereas in boar spermatozoa, it appeared also in the midpiece (30), most likely due to species-specific differences.

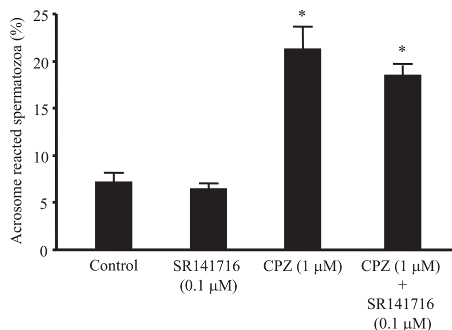

FIG. 5. Effect of SR141716 (0.1 μM), the selective CB1 antagonist, on ARs in spermatozoa capacitated for 5 h in the absence or in the presence of 1 μM CPZ. Number of replicates = 4, with different donors. Overall significance for treatment variation: $P < 0.001$ with ANOVA. *, $P < 0.05$ *vs.* control and SR141716 (Tukey's test).

Because we demonstrated here that human spermatozoa contain AEA (Table 1), and express a functional TRPV1, we explored the possible involvement of the activation of this receptor in the acquisition of sperm fertilizing ability.

In boar spermatozoa, activation of TRPV1 receptors seems to play a role in preventing spontaneous acrosome exocytosis during capacitation, because the specific antagonist of TRPV1 receptor, CPZ, highly increases the incidence of spontaneous AR while impairing a further significant AR increase in response to zona pellucida (30).

In the present study, we investigated the effect of CPZ on sperm/oocyte fusion, a relevant biological endpoint in the fertilization process. Sperm/oocyte fusion was evaluated by the P-enhanced HEPT. In the proximity of the oocyte, capacitated spermatozoa are exposed to P, a well-characterized physiological inducer of AR (51, 52), which is secreted at micromolar levels by oocyte and steroidogenic cumulus cells (53, 54). Therefore, P-enhanced HEPT evaluates the sperm ability to capacitate and to respond to P, by exhibiting a functional AR. This is accompanied by the generation of a fusogenic equatorial segment of the sperm head, which makes spermatozoa able to recognize and to fuse with the vitelline membrane of the oocyte (55). The inhibition of TRPV1 by CPZ produced a significant reduction of the stimulatory effect of P on sperm/oocyte fusion (Fig. 3). A convincing explanation of this effect is provided by the results of AR experiments. A significantly higher proportion of spermatozoa preincubated under capacitating conditions with CPZ underwent a premature acrosome exocytosis, whereas the proportion of those undergoing P-induced AR was significantly reduced (Fig. 4). Acrosome reaction is a prerequisite for successful fertilization. However, it must occur at the appropriate time and in proximity to the oocyte. Spermatozoa that have prematurely lost their acrosome are unable to fertilize oocytes (56–58). Therefore, it may be speculated that the induction of premature spontaneous acrosomal exocytosis by CPZ could reduce the proportion of fully capacitated spermatozoa able to respond to physiological inducers of functional (*i.e.* relevant to fertilization) AR, when they approach the egg. The effect of CPZ was prevented by the addition of the specific inhibitor of EMT, OMDM-1 (44), which prevented the increase in spontaneous AR rate, restoring the responsiveness to P (Fig. 5). Because AEA transport by EMT across the cell membrane is supposed to be bidirectional (45), EMT inhibition should minimize the export of AEA from spermatozoa (45), as previously reported in neurons and other cell types (59). Accordingly, in boar spermatozoa, EMT inhibition increased endogenous levels of AEA by approximately 3-fold (30). The increased intracellular AEA would displace CPZ from TRPV1, thereby preventing its inhibition of the receptor. On the other hand,

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because some of the AEA synthesized within the spermatozoa could be exported in the extracellular medium by the bidirectional EMT, any possible contribution exerted by endogenous AEA via CB1 was ruled out, because AR rate in the presence or absence of CPZ was not influenced by the coincubation with the selective CB1 antagonist SR141716 (Fig. 5). The mechanism by which the activation of TRPV1, an ion channel, could mediate its biological effects on human sperm has not been explored in this study and will be addressed in an independent investigation.

In conclusion, we show that human spermatozoa have a complete and fully active endocannabinoid system, including TRPV1. The latter receptor could be involved in the complex mechanisms that make spermatozoa able to fertilize oocytes during the capacitation process in the female genital tract.

These observations, taken together with the reported effects of extracellular AEA on CB1, seem to indicate that the endocannabinoid system exerts a complex role in modulating human sperm functions involved in the acquisition of its fertilizing ability. Anandamide, which is present both in seminal plasma and uterine fluids (32, 33), could prevent premature acrosomal loss, not functional to fertilization, as demonstrated both in boar (30) and human (34) spermatozoa. In the proximal female genital tract, spermatozoa are exposed to a progressively lower concentration of AEA (32, 33), which makes this inhibitory effect less stringent. At the same time, intracellular AEA, whose level has been demonstrated to increase during capacitation in the boar model (30), acquires a major role in preventing spontaneous ARs and in maximizing the responsiveness to physiological inducers of AR, through TRPV1. On a final note, these findings extend to human sperm the key role of the AEA-related endocannabinoid system in controlling male reproductive potential, recently demonstrated at the level of mammalian Sertoli cells (60–62), germinal cells (62, 63), and Leydig cells (62).

Acknowledgments

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