Characterization of the endocannabinoid system in boar spermatozoa and implications for sperm capacitation and acrosome reaction

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Summary

Anandamide (AEA) is the endogenous ligand of cannabinoid (CB) receptors, and as such it plays several central and peripheral activities. Regulation of female fertility by AEA has attracted growing interest, yet a role for this endocannabinoid in controlling sperm function and male fertility in mammals has been scarcely investigated. In this study we report unprecedented evidence that boar sperm cells have the biochemical machinery to bind and degrade AEA, i.e. type-1 cannabinoid receptors (CB1R), vanilloid receptors (TRPV1), AEA-synthesizing phospholipase D (NAPE-PLD), AEA transporter (AMT) and AEA hydrolase (FAAH). We also show that the non-hydrolyzable AEA analogue methanandamide reduces sperm capacitation and, as a consequence, inhibits the process of acrosome reaction (AR) triggered by the zona pellucida, according to a cyclic AMP-dependent pathway triggered by CB1R activation. Furthermore, activation of TRPV1 receptors seems to play a role of stabilization of the plasma membranes in capacitated sperm, as demonstrated by the high incidence of spontaneous AR occurring during the cultural period when TRPV1 activity was antagonized by capsazepine. We show that sperm cells have a complete and efficient endocannabinoid system, and that activation of cannabinoid or vanilloid receptors controls, at different time-points, sperm functions required for fertilization. These observations open new perspectives on the understanding and treatment of male fertility problems.

Key words: Anandamide, Cannabinoid receptor, Cyclic AMP, Metabolism, Vanilloid receptor, Zona pellucida

Introduction

Anandamide (arachidonoylethanolamide, AEA) belongs to a group of endogenous lipids, which include amides, esters and ethers of long chain polyunsaturated fatty acids, collectively termed 'endocannabinoids' (Mechoulam, 2002; De Petrocellis et al., 2004). It binds to type-1 and type-2 cannabinoid receptors (CB1R and CB2R), thus having many actions in the central (Fride, 2002) and peripheral (Parolaro et al., 2002) nervous systems. These activities of AEA are terminated by cellular uptake through an AEA membrane transporter (AMT) (Hillard and Jarrahian, 2003), followed by degradation to ethanolamine and arachidonic acid by the fatty acid amide hydrolase (FAAH) (Bisogno et al., 2002; Bracey et al., 2002). The checkpoint in AEA synthesis is the *N*-acyl-phosphatidylethanolamines (NAPE)-hydrolyzing phospholipase D (NAPE-PLD), which releases, on demand, AEA from membrane NAPEs (Okamoto et al., 2004). Together with AEA and congeners such as 2-arachidonoylglycerol (2-AG), *N*-arachidonoyldopamine, noladin ether and virodhamine, these proteins form the 'endocannabinoid system' (De Petrocellis et al., 2004). In addition, type 1 vanilloid receptors (now called transient receptor potential channel vanilloid receptor subunit 1, TRPV1), that are six trans-membrane spanning proteins with intracellular N and C termini (Jordt and Julius, 2002), have been shown to be activated by AEA (De Petrocellis et al., 2001). Therefore, this lipid is also a true 'endovanilloid' (Van der Stelt and Di Marzo, 2004).

Among the peripheral activities of AEA, regulation of fertility has attracted growing interest (Maccarrone and Finazzi-Agrò, 2004), adding the well-known effects of exogenous cannabinoids to its already established function as an endogenous ligand of CB receptors (reviewed by Park et al., 2003; Park et al., 2004). For example, low FAAH in circulating maternal lymphocytes has been shown to be an early (<8 weeks of gestation) predictor of spontaneous abortion in humans (Maccarrone and Finazzi-Agrò, 2004), and consistently FAAH expression is under control of fertility signals like progesterone and leptin (Maccarrone et al., 2003a). By contrast, mouse uterus contains the highest amounts of AEA as yet measured in any tissue (Paria and Dey, 2000), and uterine AEA can activate CB1 receptors in this organ, thus allowing epithelial changes needed for reproduction (Maccarrone and Finazzi-Agrò, 2004). Additionally, within a very narrow concentration range AEA regulates blastocyst function and implantation by differentially modulating mitogen-activated protein kinase signaling and calcium channel activity via CB1 receptors (Wang et al., 2003). Recently, it has been demonstrated that rat testis is able to synthesize AEA (Sugiura et al., 1996), and this compound has been detected also in human seminal plasma at nanomolar

concentrations (Schuel et al., 2002a). The presence of CB1 receptors in Leydig cells and their involvement in testosterone secretion have been demonstrated in mice (Wenger et al., 2001), whereas mouse Sertoli cells have been shown to possess CB2 receptors, AMT and FAAH; both these findings have led to the suggestion that the endocannabinoid network may play a role in the regulation of male fertility (Maccarrone et al., 2003b). AEA signaling has been proposed to regulate sperm functions required for fertilization in humans (reviewed in Schuel et al., 2002b), and consistently human spermatozoa have been recently shown to express CB1R at the protein and mRNA level (Rossato et al., 2005). Yet, the molecular basis of the involvement of the endocannabinoid system in controlling sperm function and male fertility in mammals remains unclear.

Chronic administration of cannabis active principle Δ^9-tetrahydrocannabinol to animals is known to induce impotence (Murphy et al., 1994), to lower testosterone secretion and to reduce the production, motility and viability of sperm (Hall and Solowij, 1998). The binding of AEA to a CB receptor present on spermatozoa of sea urchin has been shown to reduce their fertilizing capacity (Chang et al., 1993; Schuel et al., 1994), and accordingly whole homogenates of these animals were able to convert *N*-arachidonoyl-phosphatidylethanolamine (NArPE) into AEA (Bisogno et al., 1997). Differently from lower animals, ejaculated sperm from mammals must undergo a functional maturation in order to become capable of fertilizing an oocyte. Sperm acquire fertilization competence as they reside in the female genital tract where, after a series of physiological changes, they become 'capacitated' (Yanagimachi, 1994). Therefore, capacitation consists of changes occurring on the sperm head that enable it to bind to the zona pellucida and undergo the acrosome reaction, and in the flagellum, where hyperactivated sperm motility is facilitated. The control of this crucial process involves modifications of intracellular ions (Fukami et al., 2003; Wennemuth et al., 2003), of plasma membrane fluidity (Harrison, 1997; Gadella and Harrison, 2000), and of metabolism and motility (Suarez and Ho, 2003). However, the sequence of these changes, and in particular the local regulatory mechanisms that allow capacitation to progress as the sperm become closer to the oocyte, still remain poorly understood. During the preparation of this manuscript an interesting study has appeared, showing that AEA reduces human sperm motility by reducing mitochondrial activity, and that it also inhibits capacitation-induced acrosome reaction (Rossato et al., 2005). These effects of AEA were prevented by the CB1R antagonist SR141716, leading to the suggestion that they required CB1R activation (Rossato et al., 2005). However, the functionality of CB1R in human sperm was not ascertained, neither was the ability of these cells to bind, synthesize, transport and degrade AEA. Yet, the analysis of these components of the endocannabinoid system is of utmost importance, because converging evidence suggests that AEA signaling and biological activity is tightly regulated via a 'metabolic control' (Bisogno et al., 2002; Bracey et al., 2002; Park et al., 2004). With this knowledge we sought to investigate whether sperm cells of boar (*Sus scropha*) were able to bind and metabolize AEA, and whether this endocannabinoid might modulate their function. It should be recalled that boar physiology closely resembles that of humans (Logan and Sharma, 1999), and its spermatozoa are largely used as a model system for experiments on reproductive physiology of mammals (Gadella et al., 1995; James et al., 2004). However, we did not further extend this study to the metabolic enzymes that degrade (Dinh et al., 2002) and synthesize (Bisogno et al., 2003) 2-AG, because the properties of these recently discovered proteins are still under investigation.

Materials and Methods

Materials

Chemicals were of the purest analytical grade. Anandamide (arachidonoylethanolamide, AEA) and resinferatoxin (RTX) were from Sigma Chemical Co. (St Louis, MO, USA). R(+)-methanandamide (Met-AEA), capsaicin and capsazepine (*N*-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbothioamide; CPZ) were from Calbiochem (San Diego, CA, USA), and VDM11 was from Tocris-Cookson (Bristol, UK). [^{3}H]AEA (223 Ci/mmol), [^{3}H]CP55.940 (126 Ci/mmol), and [^{3}H]RTX (43 Ci/mmol) were purchased from Perkin Elmer Life Sciences (Boston, MA, USA). *N*-[^{3}H]arachidonoyl-phosphatidylethanolamine ([^{3}H]NArPE; 200 Ci/mmol) was from ARC (St Louis, MO, USA). *N*-Piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole carboxamide (SR141716) and *N*-[1(*S*)-endo-1,3,3-trimethyl-bicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methyl-benzyl)-pyrazole-3-carboxamide (SR144528) were kind gifts from Sanofi-Aventis Recherche (Montpellier, France). Rabbit anti-CB1R and anti-CB2R polyclonal antibodies were from Cayman Chemicals (Ann Arbor, MI, USA), whereas rabbit anti-TRPV1 polyclonal antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Rabbit anti-FAAH polyclonal antibodies (Maccarrone et al., 2001) were prepared by Primm S.r.l. (Milan, Italy). Goat anti-rabbit alkaline phosphatase conjugates (GAR-AP) were from Bio-Rad (Hercules, CA, USA), goat anti-rabbit CY3-conjugated IgGs were from Sigma, and Alexa FluorResting® 488 donkey anti-goat IgGs was from Molecular Probes (Eugene, OR, USA).

The reagents used for in vitro experiments such as bovine serum albumin (BSA), TCM 199, polyvinylpyrrolidone (PVP-40), chlortetracycline (CTC), Percoll, lectin from *Pisum sativum* conjugated with fluorescein isothiocyanate (FITC-PSA), bisbenzimide H 33258 (Hoechst 33258), mounting medium, normal goat serum, Triton X-100 and Tween 20 were purchased from Sigma. 8-Bromoadenosine 3′,5′ cyclic monophosphate (8Br-cAMP) was from Calbiochem (San Diego, CA, USA), and the Biotrak cAMP enzyme immunoassay system (RPN 225) was purchased from Amersham (Little Chalfont, Bucks, UK).

Sperm preparation

Semen samples were collected from three boars of proven fertility before each experiment. The seminal plasma was removed by centrifugation through a two-step discontinuous gradient of 35% and 70% isotonic Percoll (Harrison, 1996), to select motile cells. After removal of the supernatant layers, the resultant loose pellet was resuspended in residual 70% Percoll and washed by centrifugation at 800 ***g*** for 10 minutes in Dulbecco's medium with Ca^{2+} and Mg^{2+}. BSA was omitted, because it is known to bind endocannabinoids (De Petrocellis et al., 2001; Ross et al., 2001; Parolaro et al., 2002; Hillard and Jarrahian, 2003), thus impairing biochemical and functional assays. The sperm sample was divided into two portions and both pellets were diluted in TCM 199 supplemented with 1.25 mM calcium lactate, 1.25 mM sodium pyruvate and 13.9 mM glucose, to a final concentration of 2×10^8 spermatozoa/ml. The first portion was used for biochemical and immunocytochemical analysis, the second was used for in vitro

capacitation at 38.5°C in 5% CO_2 humidified atmosphere for up to 4-6 hours.

Assessment of sperm viability

At the beginning of the culture, sperm cells were ~90% viable. This value was progressively reduced during the incubation period, but only samples showing mean viability of ~70% were used for further analysis. Sperm viability was assessed at the end of each culture period, using Hoechst 33258 (Barboni et al., 1995; Wang et al., 1995). We did not assess sperm motility during the study, because this functional parameter is known to be impaired during the incubation in culture media devoid of BSA (Harrison et al., 1978; Harrison et al., 1982). Consistently, in our preliminary experiments a progressive motility was maintained by only 40-50% of spermatozoa analyzed after 4 hours of culture. Samples of capacitated spermatozoa used for biochemical analysis were further selected at the end of the culture period using a Percoll gradient, as previously described, to eliminate dead spermatozoa (Harrison, 1996).

Assays of the endocannabinoid system

Cannabinoid receptor (Maccarrone et al., 2000) and vanilloid receptor (Ross et al., 2001) studies were performed on freshly isolated sperm cells (500×10^6/test) by rapid filtration assays, using [^{3}H]CP55.940 and [^{3}H]RTX, respectively. Apparent dissociation constant (K_d) and maximum binding (B_{max}) values were calculated from saturation curves through nonlinear regression analysis with the Prism 3 program (GraphPAD Software for Science, San Diego, CA, USA) (Maccarrone et al., 2000). Binding of [^{3}H]AEA was performed on the same membrane preparations and with the same filtration assays used for [^{3}H]CP55.940. In addition, data for saturation curves of [^{3}H]CP55.940 binding to CB1 and CB2 receptors on mouse brain and mouse spleen extracts were obtained using the same experimental conditions. The expression of CB1, CB2 and TRPV1 receptors in boar sperm was assessed by western blot analysis, using anti-CB1R, anti-CB2R (each diluted 1:250) or anti-TRPV1 (1:500) polyclonal antibodies, and GAR-AP (diluted 1:2000) as the secondary antibody (Maccarrone et al., 2001). Non-immune rabbit serum (Primm S.r.l.) was used as a control for specificity of the polyclonal antibodies (Maccarrone et al., 2001).

The uptake of [^{3}H]AEA by the AEA membrane transporter (AMT) of intact sperm cells (10×10^6/test) was performed as described previously (Maccarrone et al., 2002). The apparent Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) of AMT were determined by nonlinear regression analysis. The synthesis of [^{3}H]AEA through the activity of *N*-acyl-phosphatidylethanolamines (NAPE)-hydrolyzing phospholipase D (E.C. 3.1.4.4; NAPE-PLD) was assayed in sperm cell homogenates (50 μg/test), using 100 μM [^{3}H]NArPE as reported previously (Okamoto et al., 2004). The hydrolysis of [^{3}H]AEA by the fatty acid amide hydrolase (E.C. 3.5.1.4; FAAH) activity was assayed in sperm cell extracts (20 μg/test) by measuring the release of [^{3}H]arachidonic acid from [^{3}H]AEA through reversed phase high performance liquid chromatography (HPLC), as reported previously (Maccarrone et al., 2000). Apparent K_m and V_{max} values of FAAH were determined as described above for AMT. FAAH expression was determined in cell homogenates (20 μg/lane) as described previously (Maccarrone et al., 2001), using bovine serum albumin (92 kDa), ovalbumin (52 kDa) and carbonic anhydrase (36 kDa) as molecular mass markers (Bio-Rad). FAAH protein content was quantified by enzyme-linked immunosorbent assay (ELISA), performed on cell homogenates (20 μg/well) as reported previously (Maccarrone et al., 2001).

For the evaluation of endogenous AEA levels, sperm cells (1×10^9/test) were homogenized with an Ultra Turrax T25 in 50 mM Tris-HCl, 1 mM EDTA pH 7.4 and 1 mM phenylmethanesulfonyl fluoride buffer, at a 1:10 (wt/vol) homogenization ratio. Lipids were then extracted (Maccarrone et al., 2000) and the organic phase was dried under nitrogen. Dry pellet was resuspended in 20 μl of methanol, and was processed and analyzed by HPLC with fluorimetric detection as reported previously (Wang et al., 2001).

Detection of sperm capacitation

Sperm capacitation in vitro was determined by the ability of the spermatozoa to display chlortetracycline (CTC) fluorescence 'pattern B', indicative of the capacitative status (Wang et al., 1995; Mattioli et al., 1996; Maxwell and Johnson, 1997). In addition, a set of experiments was carried out to evaluate the competence of in vitro capacitated spermatozoa to undergo acrosomal exocytosis in response to solubilized zonae pellucidae (ZP), as a functional endpoint of the capacitative state. With this aim, in vitro capacitated sperm were exposed for 30 minutes to solubilized ZP and the resulting total acrosome reaction (AR) was detected by FITC-PSA staining (Ward and Storey, 1984; Barboni et al., 1995). PSA has been shown to bind selectively to the acrosomal content on the sperm head in a number of species [human (Cross et al., 1986); monkey (Cross et al., 1989); pig (Barboni et al., 1995)]. Intense staining of the acrosomal content, with much less label on the other regions of the cell, is indicative of an intact acrosome. Sperm that have undergone the AR and have lost most or all of the acrosomal content, bind much less PSA and can be detected by the faint fluorescence of the anterior head.

The spermatozoa were fixed and permeabilized for at least 30 minutes at 4°C in 95% (v/v) ethanol, to allow entry of the PSA. Permeabilized sperm, dried onto microscope slides, were then covered with a droplet of FITC-conjugated PSA (100 mg/ml in PBS) for 10 minutes. After repeated washing of the slide in bi-distilled water, the sperm were analyzed under a fluorescence microscope. In each case sperm head scoring was carried out in pairs until 400 live spermatozoa, 200 on each slides, were analyzed. Since spermatozoa incubated for in vitro capacitation spontaneously and variably excogitate their acrosomal content in the absence of any specific stimulus, any acrosomal exocytosis before exposing spermatozoa to ZP was considered as 'spontaneous AR'. This spontaneous AR was subtracted from total AR, obtained after exposure for 30 minutes to solubilized ZP, in order to obtain the incidence of the true ZP-induced AR.

Measurement of intracellular cAMP content

Spermatozoa (300 μl/sample) incubated under capacitating conditions for 4 hours were pelleted at 500 ***g*** for 5 minutes, and then frozen at –80°C until the assay was carried out using a Biotrak cAMP enzyme immunoassay system (RPN 225). The optical density of the samples was determined at 450 nm in a BioRad plate reader.

Protein visualization by cytochemistry

For CB1R analysis, freshly isolated sperm samples were washed in PBS, fixed in 0.5% glutaraldehyde supplemented with 1% paraformaldehyde in 0.1 mM cacodilate buffer, pH 7.4, for 1 hour at 4°C. Then, they were incubated with rabbit anti-CB1R antibodies (diluted 1:800) for 2 hours at room temperature (RT), and with goat anti-rabbit CY3 conjugates (1:400) for 1 hours at room temperature (RT). Fresh spermatozoa used for the detection of TRPV1 and FAAH, which are intracellular proteins not accessible to antibodies in intact cells (De Petrocellis et al., 2001; Jordt and Julius, 2002; Oddi et al., 2005), were fixed and permeabilized with Triton X-100 (0.5% in PBS, plus 1% BSA) for 30 minutes at RT. Washed spermatozoa were then resuspended in normal goat serum (20%) for 30 minutes at RT, and were then exposed at 38°C for 1 hour to goat anti-TRPV1 (1:400) or rabbit anti-FAAH (1:100) antibodies, diluted in PBS containing 1% BSA and 0.05% Tween 20. After three centrifugation steps in PBS with 0.1% BSA (800 ***g***, 5 minutes), spermatozoa were incubated for

1 hour at RT with antibodies conjugated to Alexa Fluor® 488 (donkey anti-goat IgGs; dilution 1:400) or to Cy43 (goat anti-rabbit IgGs; dilution 1:400), for TRPV1 and FAAH, respectively. Also these conjugates were resuspended in PBS supplemented with 0.1% BSA and 0.05% Tween 20. All immunocytochemical analyses were performed in the absence of primary antibodies. In addition, non-permeabilized spermatozoa were used as negative controls for the intracellular proteins FAAH and TRPV1. The images were acquired using a Nikon Eclipse E600 microscope, equipped with a Nikon Plan Fluor 100× objective, a EX330-380 DM400 BA420 filter, a EX465-495 DM505 BA420 filter, a EX510-560 DM 575 BA590 filter and a Nikon DMX1200 digital camera provided with ACT-1 software for DXM1200.

Statistical analysis

Data reported in this paper are the mean (± s.d.) of at least three independent determinations, each in duplicate. Statistical analysis was performed by the nonparametric Mann-Whitney U test, elaborating experimental data by means of the InStat 3 program (GraphPAD Software for Science). Differences were considered significant and highly significant for P values of <0.05 and <0.01, respectively.

Results

AEA-binding receptors in sperm cells

Boar sperm cells were able to bind the synthetic cannabinoid [^{3}H]CP55.940, which has high affinity to both CB1 and CB2 receptors (Pertwee, 1997), according to a saturation process (Fig. 1A). The selective CB1R antagonist SR141716, but not the selective CB2R antagonist SR144528 (Pertwee, 1997), displaced [^{3}H]CP55.940 suggesting that only CB1 receptors were expressed on sperm cell surface (Fig. 1B). In addition, AEA and its non-hydrolyzable analogue methanandamide (Met-AEA), which are equally potent CBR agonists (Lin et al., 1998) and have higher affinity for CB1R than for CB2R (Palmer et al., 2002), almost completely displaced [^{3}H]CP55.940 binding (Fig. 1B). In order to further confirm the presence of CB1 receptors in these cells, saturation curves of [^{3}H]CP55.940 binding were compared to those in mouse brain (a positive control for CB1R) and in mouse spleen (a positive control for CB2R) (Pertwee, 1997; Lin et al., 1998). [^{3}H]CP55.940 was found to bind to sperm cell membranes with saturation curves very close to those obtained with mouse brain membranes (Fig. 1A). From these curves K_d values of 704±103 and 598±86 pM, and B_{max} values of 1682±119 and 1773±115 fmol/mg protein, could be calculated for sperm cells and brain. respectively. The K_d and B_{max} values of boar spermatozoa are in keeping with those reported for sea urchin and human sperm (Chang et al., 1993; Schuel et al., 2002b). On the other hand, binding of [^{3}H]CP55.940 to mouse spleen (Fig. 1A) showed a K_d of 245±30 pM and a B_{max} of 276±10 fmol/mg protein. Incidentally, the K_d and B_{max} values found here for mouse brain and spleen are in agreement with previous reports (reviewed by Pertwee, 1997). Consistently with the binding data, western blot analysis showed that in sperm cell extracts specific anti-CB1R antibodies recognized a single immunoreactive band at the expected molecular size of CB1R (~56 kDa), whereas specific anti-CB2 antibodies yielded only a weak staining at the expected molecular size (~48 kDa) of CB2R (Fig. 2A). Moreover, sperm cells were able to dose-dependently bind [^{3}H]RTX, a specific TRPV1 agonist (Zygmunt et al., 1999; Ross et al., 2001), and 1 μM capsazepine (CPZ), a selective antagonist of TRPV1 receptors (Zygmunt et al., 1999; Ross et al., 2001), fully displaced this binding (Fig. 1C). Also AEA reduced the binding

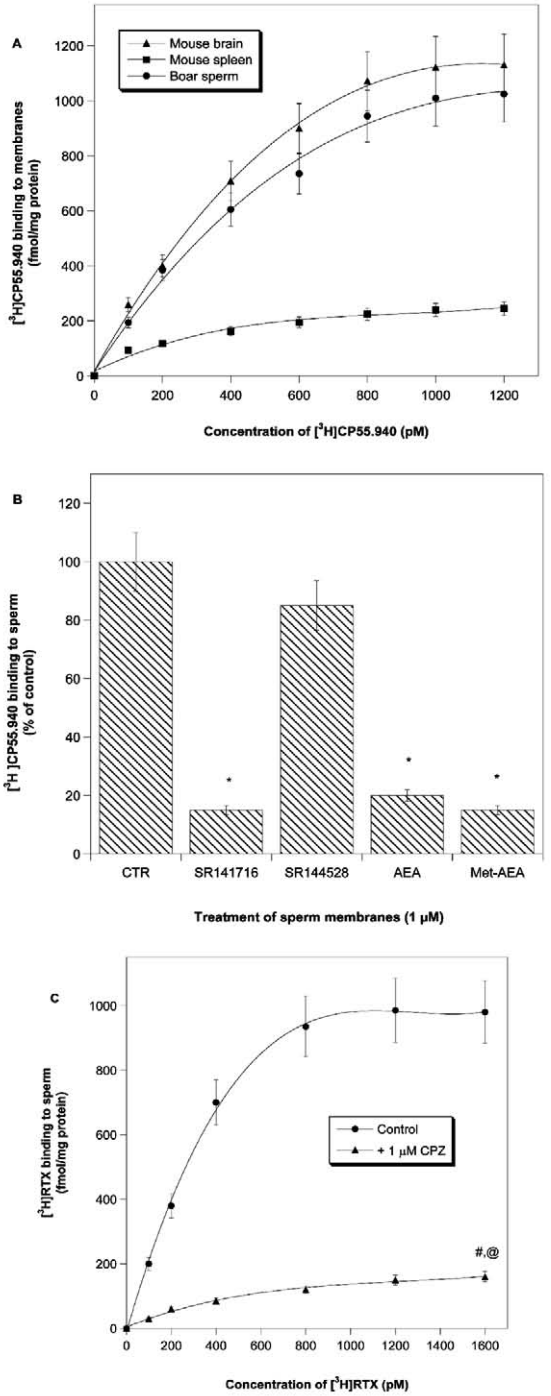

Fig. 1. Cannabinoid and vanilloid receptors in sperm cells. (A) Saturation curves of [^{3}H]CP55.940 binding to mouse brain, mouse spleen or boar sperm membranes. (B) Effect of CB1 and CB2 receptor antagonists SR141716 and SR144528, and of natural (AEA) or synthetic (Met-AEA) agonists on the binding of 400 pM [^{3}H]CP55.940 (100% as in A). *P<0.01 versus control (CTR). (C) Saturation curves of [^{3}H]RTX binding to boar sperm membranes, alone or in the presence of 1 μM CPZ. The effect of 1 μM AEA (#) or 1 μM Met-AEA (@) on the binding of 1600 pM [^{3}H]RTX is also shown. In all panels, vertical bars represent the s.d.

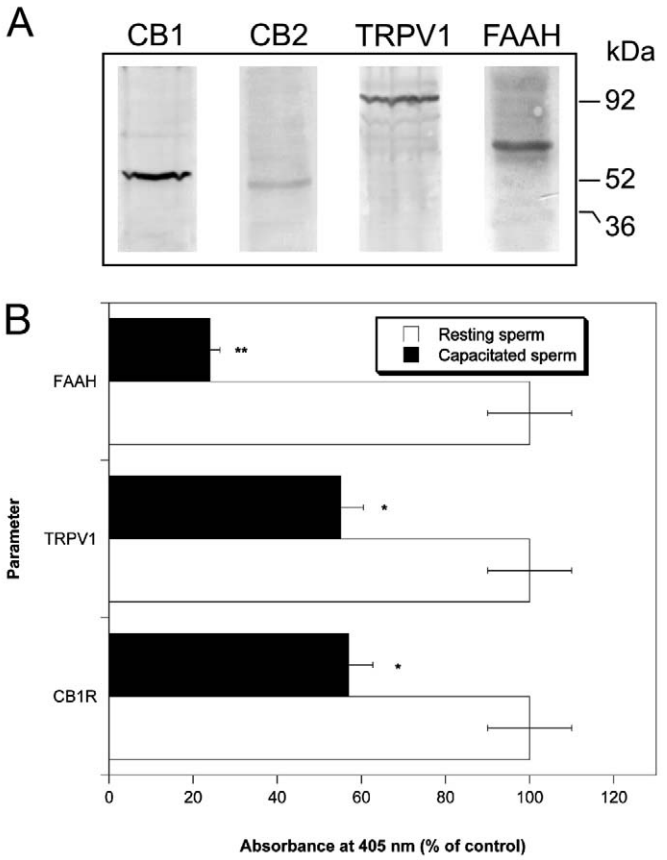

Fig. 2. Expression of CB1, CB2 and TRPV1 receptors, and FAAH, in sperm cells. (A) Western blot analysis of sperm cell extracts (20 μg/lane) reacted with specific polyclonal antibodies. Gels were overstained in order to check for antibody specificity. Molecular mass markers are shown on the right side. (B) ELISA test of resting or capacitated sperm cells, reacted with the same antibodies as in A. Values were expressed as percentage of the resting cells, set to 100 (100%=0.340±0.040, 0.270±0.030, or 0.300±0.030 absorbance units at 405 nm for CB1R, TRPV1 or FAAH, respectively). In B, horizontal bars represent the s.d.; *P<0.05, **P<0.01 versus resting sperm cells.

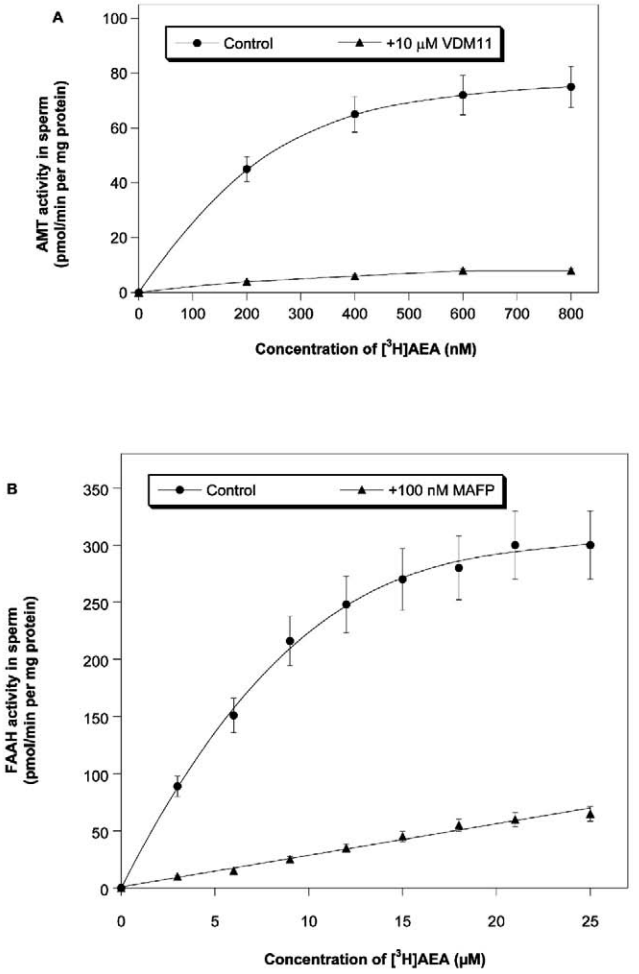

Fig. 3. AMT and FAAH activity in sperm cells. (A) Dependence of AMT activity on AEA concentration, alone or in the presence of the AMT inhibitor VDM11 at 10 μM. (B) Dependence of FAAH activity on AEA concentration, alone or in the presence of the FAAH inhibitor MAFP at 100 nM. In both panels, vertical bars represent the s.d.

of 1600 pM [^{3}H]RTX down to ~20% of the controls, when used at a concentration of 1 μM (Fig. 1C). This finding is in keeping with the notion that AEA is a true endovanilloid (De Petrocellis et al., 2001; Van der Stelt and Di Marzo, 2004). In addition, 1 μM Met-AEA had the same effect as AEA under the same experimental conditions (Fig. 1C). Kinetic analysis of saturation curves like that shown in Fig. 1C yielded K_d and B_{max} values of 419±91 pM and 1331±102 fmol/mg protein respectively, demonstrating that the affinity of RTX binding to sperm cells is typical of authentic TRPV1 receptors (Ross et al., 2001). Western blot analysis corroborated the binding data, showing that in sperm cell extracts specific anti-TRPV1 antibodies recognized a single immunoreactive band (Fig. 2A) of the expected molecular size (~95 kDa) of TRPV1 (Ross et al., 2001). Finally, sperm cells were able to bind [^{3}H]AEA (450±50 c.p.m. upon incubation with 200 nM [^{3}H]AEA), and this binding was fully displaced by 1 μM SR141716 + 1 μM CPZ, whereas 1 μM CPZ alone displaced ~50% of bound [^{3}H]AEA under the same experimental conditions.

AEA metabolism and endogenous levels in sperm cells

Intact sperm cells were able to accumulate [^{3}H]AEA according to a saturable process (Fig. 3A), that is typical of AMT (Maccarrone et al., 2002; Hillard and Jarrahian, 2003). Apparent Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) values of [^{3}H]AEA uptake were 220±30 nM and 97±4 pmol/minute per mg protein respectively, in keeping with the kinetic constants of authentic AMT (Maccarrone et al., 2002; Hillard and Jarrahian, 2003). In addition, 10 μM VDM11, a specific AMT inhibitor (De Petrocellis et al., 2001), fully inhibited [^{3}H]AEA uptake by sperm cells (Fig. 3A).

Sperm cells showed also FAAH activity, which hydrolyzed [^{3}H]AEA dose-dependently with apparent K_m and V_{max} values of 10±1 μM and 443±25 pmol/minute per mg protein (Fig. 3B). 100 nM MAFP, a specific FAAH inhibitor (De Petrocellis et al., 2001), completely blocked [^{3}H]AEA hydrolysis (Fig. 3B). Kinetic data were further confirmed by western blot analysis of sperm cell extracts, showing that specific anti-FAAH antibodies recognized a single immunoreactive band of the molecular size (~68 kDa) expected for FAAH (Fig. 2A).

Table 1. The endocannabinoid system in resting and capacitated sperm cells

Parameter	Resting sperm cells	Capacitated sperm cells
CB1R binding[†]	600±50 (100%)	360±38 (60%)*
TRPV1 binding[‡]	700±60 (100%)	406±42 (58%)*
AMT activity[§]	65±7 (100%)	33±4 (51%)**
FAAH activity[¶]	220±20 (100%)	20±5 (9%)**
NAPE-PLD activity[††]	45±5 (100%)	29±4 (64%)*
Endogenous AEA level[‡‡]	0.03±0.01 (100%)	0.06±0.01 (200%)**

[†]Expressed as fmol/mg protein (substrate was 400 pM [^{3}H]CP55.940).
[‡]Expressed as fmol/mg protein (substrate was 400 pM [^{3}H]RTX).
[§]Expressed as pmol/minute per mg protein (substrate was 400 nM [^{3}H]AEA).
[¶]Expressed as pmol/minute per mg protein (substrate was 10 μM [^{3}H]AEA).
[††]Expressed as pmol/minute per mg protein (substrate was 100 μM [^{3}H]NArPE).
[‡‡]Expressed as pmol/mg protein.
*P<0.05, **P<0.01 versus corresponding control.

The lack of anti-AMT antibodies prevented the extension of the western blot analysis to the AEA transporter.

Sperm also showed NAPE-PLD activity, and in fact endogenous AEA was detected in these cells (Table 1). Additionally, AEA was detected also in boar seminal plasma, at a concentration (1.53±0.30 pmol/ml) in the same nanomolar range recently detected in human seminal plasma (Schuel et al., 2002a). The lack of specific inhibitors of NAPE-PLD (Okamoto et al., 2004) and of anti-NAPE-PLD antibodies prevented inhibition experiments and western blot analysis on this protein.

Visualization of CB1R, TRPV1 and FAAH in sperm cells

Immunocytochemical analysis revealed that CB1R, TRPV1 and FAAH were clearly detectable on the sperm head, where they seemed confined in the post-acrosomal region (Fig. 4). Moreover, fresh spermatozoa displayed positive signals for CB1R, TRPV1 and FAAH in the middle region, and additionally TRPV1 and FAAH could be clearly detected only in sperm cells with permeabilized membranes (data not shown). The latter finding is in keeping with the intracellular localization of TRPV1 binding site for AEA (De Petrocellis et al., 2001; Jordt and Julius, 2002) and of FAAH (Oddi et al., 2005).

The endocannabinoid system in capacitated sperm cells

The endocannabinoid system was also analyzed in capacitated sperm cells, and the results are shown in Table 1 and Fig. 2B. CB1R and TRPV1 binding, and the activity of AMT, FAAH and NAPE-PLD were all significantly reduced in capacitated cells compared with the controls (Table 1). In particular, FAAH activity of capacitated cells was less than 10% of the activity of controls, whereas NAPE-PLD activity was reduced to 64% only (Table 1). Consistently, endogenous AEA content was doubled in capacitated sperm compared with controls (Table 1). The same antibodies used in western blot analysis were used to quantify CB1R, TRPV1 and FAAH content in resting compared with capacitated cells by ELISA, showing that all proteins were down-regulated upon capacitation (Fig. 2B). The reduction of protein expression could fully account for reduced CB1R binding and NAPE-PLD activity, whereas it only partly accounted for the reduction of FAAH activity (compare Table 1 and Fig. 2B).

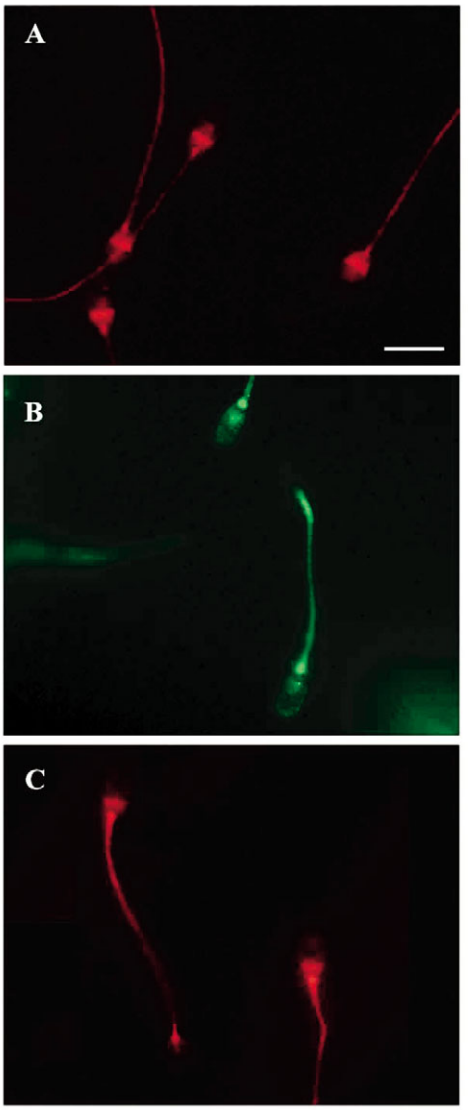

Fig. 4. Immunocytochemical analysis of CB1R receptors (A), TRPV1 receptors (B) and FAAH (C), in ejaculated boar sperm cells. Both receptors and the AEA hydrolase FAAH are localized on the post acrosomal region of the sperm head and on the whole middle region. Scale bar: 1 μm.

Role of CB1R on sperm function

Since sperm cells efficiently degrade AEA through AMT and FAAH (Fig. 3A,B), we chose the non-hydrolyzable AEA analogue Met-AEA to further investigate the activity of endocannabinoids on sperm function. It should be noted that the receptor binding properties of Met-AEA resemble those of AEA (Fig. 1B,C), as already described for CB1R (Lin et al., 1998) and TRPV1 (Ralevic et al., 2001).

Treatment with Met-AEA reduced sperm capacitation in a time-dependent manner. Fig. 5 shows that the percentage of

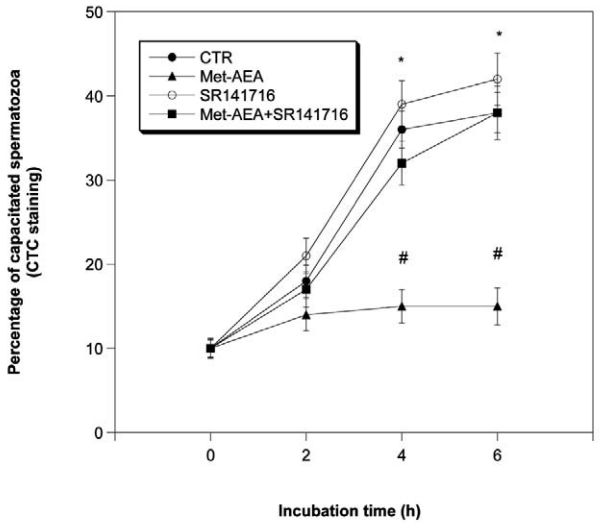

Fig. 5. Time-dependent changes of capacitation, assessed by chlortetracycline (CTC) staining, in spermatozoa incubated for 6 hours in the absence (CTR) or presence of 1 μM Met-AEA, 0.1 μM SR141716 (the CB1R antagonist) or both. Standard deviation values range amongst experiments from ±2.0 to ±3.1. **P*<0.01 versus CTR at time 0; #*P*<0.01 versus CTR at the corresponding time point.

capacitated sperm, as indicated by CTC 'pattern B', progressively increased during the incubation period, and reached a plateau after 4 hours of culture (36.0±2.2% and 38.6±2.4% after 4 and 6 hours in culture, respectively). In the presence of 1 μM Met-AEA the spermatozoa displayed a reduced ability to capacitate, and in fact the percentage of spermatozoa with pattern B remained stable during the whole incubation period (10% at the beginning versus 15.2±2.2% at the end of the culture; *P*>0.05; see Fig. 5). The inhibitory effect of 1 μM Met-AEA was completely removed by the selective CB1R antagonist SR141716, which brought the CTC-fluorescence pattern 'B' back to the control level at the end of the culture (38.4±3.1%; *P*>0.05 versus control group; see Fig. 5). Moreover, SR141716 per se did not affect sperm capacitation in vitro (Fig. 5).

To further confirm the status of capacitation and to assess the function of the cell machinery involved in acrosome exocytosis, a second set of experiments was carried out to evaluate the ability of in vitro-capacitated spermatozoa to undergo AR in response to a physiological stimulus such as solubilized ZP. Moreover, the incidence of AR obtained in vitro in the absence of any physiological stimulus (i.e. spontaneous AR) was also recorded. As shown in Fig. 6A, the percentage of spontaneous AR, during the incubation period (4 hours), ranged between 12% and 15%, independent of the treatment. Instead, in the presence of ZP, 42.0±3.1% of spermatozoa capacitated in vitro underwent total AR, yielding a percentage of true ZP-induced AR of 29.0±1.1%. The inhibitory role of Met-AEA (1 μM) on sperm capacitation was confirmed by the significant reduction (approx. threefold) of the ZP-induced AR. In addition, the inhibitory role of Met-AEA was again removed by co-incubation with 1 μM SR141716 (31.4±1.5; *P*>0.05 versus control group; see Fig. 6A).

Since a major CB1R-dependent signaling pathway involves down-regulation of adenylate cyclase activity (Di Marzo et al., 2002; Howlett et al., 2002), and cAMP has been shown to be an important regulator of sperm capacitation (Harrison, 2004), this secondary messenger was measured in in vitro-capacitated spermatozoa. Treatment of spermatozoa with 1 μM Met-AEA significantly reduced intracellular cAMP, to ~40% of the control values. Consistent with the activation of CB1R, the addition of 1 μM SR141716 removed the inhibitory effect of Met-AEA on the cAMP intracellular content (Table 2). In addition, when capacitation was carried out in the presence of the cAMP-permeable analogue 8Br-cAMP (1 mM), 1 μM Met-AEA was unable to prevent capacitation, as indicated by the incidence of true AR (10.7±2.2% and 21.0±1.2% in Met-AEA versus 8 Br-cAMP plus Met-AEA, respectively: *P*<0.01;

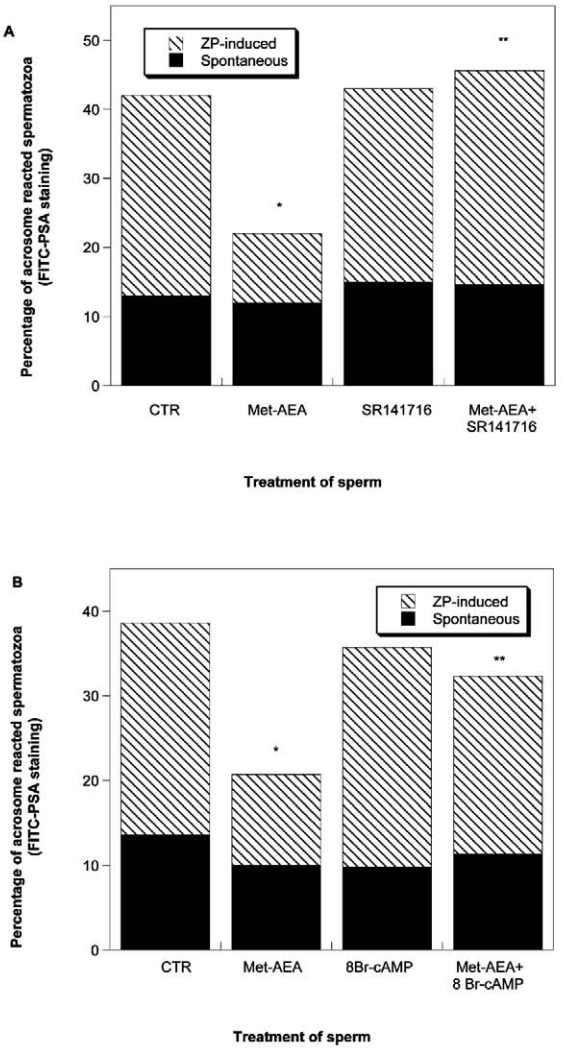

Fig. 6. Effect of Met-AEA on spontaneous and zona pellucida (ZP)-induced acrosome reaction (AR) detected by FITC-PSA staining. (A) Percentage of AR in spermatozoa capacitated in vitro for 4 hours with 1 μM Met-AEA, 0.1 μM SR141716 or both. (B) Spontaneous and ZP-induced AR recorded in spermatozoa incubated in the absence or presence of 1 μM Met-AEA, 1 mM 8Br-cAMP or both. In A and B the maximum standard deviation was ±1.5 and ±2.0, respectively; **P*<0.01 versus CTR, ***P*<0.01 versus Met-AEA for ZP-induced AR. Samples were not statistically different (*P*>0.05) for spontaneous AR.

Table 2. Effect of Met-AEA on intracellular levels of cAMP in sperm cells

Sample	cAMP (fmol/10^6 cells)†		
	Donor 1	Donor 2	Donor 3
Control	824±202 (100%)	1676±318 (100%)	953±180 (100%)
+0.1 μM SR141716	830±180 (101%)	1690±320 (101%)	1000±189 (105%)
+1 μM Met-AEA	231±134 (28%)*	498±174 (30%)*	543±168 (57%)**
+1 μM Met-AEA +0.1 μM SR141716	800±145 (97%)‡	1500±270 (89%)‡	890±163 (93%)‡

†Levels of cAMP were determined 4 hours after treatment.
*P<0.01, **P<0.05 versus control, ‡P<0.01 versus Met-AEA-treated cells.

see Fig. 6B). The latter value was similar to that observed in controls (25.1±2.0%; P>0.01).

Role of AMT and TRPV1 in sperm function

Treatment of sperm cells for 4 hours with 1 μM capsazepine (CPZ), a selective antagonist of TRPV1 receptors (Zygmunt et al., 1999), slightly affected the process of capacitation in vitro detected by CTC staining (29.9±4.2% and 36.0±2.2% of spermatozoa with pattern B, in CPZ and control groups, respectively; P>0.05). In the presence of 1 μM CPZ, incubation was remarkably characterized by a high incidence (approx. threefold) of spontaneous AR (12.1±0.9 and 35.2±1.1%, in control and CPZ-treated spermatozoa, respectively; P<0.01). As a consequence, when CPZ-treated spermatozoa were exposed to ZP only 5% of them underwent true AR (32.0±1.1% in control spermatozoa; P<0.01; see Fig. 7).

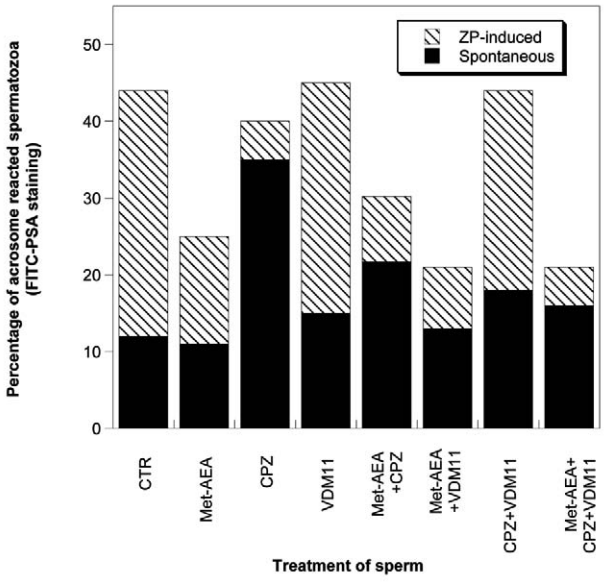

Fig. 7. Role of AMT and TRPV1 in acrosome reaction (AR). Spontaneous or ZP-induced AR recorded in spermatozoa capacitated in vitro for 4 hours in the absence (CTR) or presence of 1 μM Met-AEA, 1 μM CPZ, 10 μM VDM11 or their combinations. Standard deviation values ranged amongst experiments from 0.8 to 3.6. Statistical analysis of spontaneous AR data revealed the following: P<0.01 for CTR versus CPZ, CPZ versus CPZ plus VDM11, and CPZ versus CPZ plus VDM11 plus Met-AEA; P<0.05 for CTR versus Met-AEA plus CPZ, and CPZ versus Met-AEA plus CPZ. Statistical analysis of ZP-induced AR revealed the following: P<0.01 for CTR versus Met-AEA, CTR versus CPZ, CTR versus Met-AEA plus CPZ, CTR versus Met-AEA plus VDM11, and CTR versus Met-AEA plus CPZ plus VDM11.

Interestingly, 10 μM VDM11, a specific AMT inhibitor (De Petrocellis et al., 2001), minimized the effects of 1 μM CPZ by reducing the spontaneous AR (to 18.2±0.8% of the total population; P<0.01 versus CPZ) during the culture period. In parallel, spermatozoa re-acquired the ability to react to the physiological stimulus and showed a percentage of ZP-induced AR similar to that recorded under control conditions (Fig. 7). In order to clarify the effect of VDM11, endogenous levels of AEA were measured in sperm cells treated with 10 μM VDM11 for 4 hours. Under these experimental conditions, intracellular AEA levels were found to be approx. threefold higher than those of controls (0.10±0.01 and 0.03±0.01 pmol/mg protein, respectively), suggesting that increased intracellular AEA in VDM11-treated cells might displace CPZ from the binding site of TRPV1. The effect of CPZ on spontaneous AR was also significantly reduced by the simultaneous presence of 1 μM Met-AEA (21.7±0.8%; P<0.05 versus CPZ alone and P>0.05 versus CTR sample). This result is more likely a consequence of the inhibitory role of Met-AEA on sperm capacitation through CB1R than a consequence of the stimulatory effect of increased intracellular concentration of Met-AEA on TRPV1. In fact, spermatozoa capacitated both with Met-AEA plus CPZ and with Met-AEA plus CPZ and VDM11, even if they showed a significant reduction in the incidence of spontaneous AR, failed to undergo AR when stimulated by ZP (Fig. 7). In the same way, 1 μM capsaicin, a plant-derived agonist of TRPV1 that does not bind to CB1R (Di Marzo et al., 2002), failed to reduce the effect of CPZ on spontaneous AR.

Discussion

In this investigation we report unprecedented evidence that boar sperm have the biochemical machinery to bind (CB1R and TRPV1), synthesize (NAPE-PLD) and degrade (AMT and FAAH) AEA. We also show that activation of CB1R by the AEA stable analogue Met-AEA inhibits capacitation and hence the ability of sperm cells to react to zona pellucida proteins with acrosome exocytosis, through a cAMP-dependent pathway. In addition we demonstrate that, once sperm have completed capacitation, AEA appears to stabilize the acrosome membranes by activating TRPV1 receptors.

Sperm cells express functional type-1 cannabinoid (CB1) receptors on their surface, as suggested by: (i) saturation curves of [^{3}H]CP55.940 (Fig. 1A) and displacement of this radioligand by SR141716 (Fig. 1B), (ii) K_d and B_{max} values calculated from binding curves (Fig. 1A) and (iii) cross-reactivity with specific anti-CB1R antibodies (Fig. 2A). Altogether, these data provide further biochemical background to previous investigations that showed that human testis expresses CB1R transcripts (Gerard et al., 1991), and that human spermatozoa bind [^{3}H]CP55.940 dose-dependently, through a non-characterized receptor (Schuel et al., 2002b). In addition, they give biochemical support to a recent report,

which appeared during the preparation of this manuscript, showing CB1R protein and mRNA in human sperm (Rossato et al., 2005). Interestingly, sperm cells also express TRPV1 receptors, as demonstrated by: (i) saturation curves of [^{3}H]RTX and displacement of this radioligand by CPZ (Fig. 1C), (ii) K_d and B_{max} values calculated from binding curves (Fig. 1C), and (iii) cross-reactivity with specific anti-TRPV1 antibodies (Fig. 2A). To our knowledge, this is the first report showing TRPV1 receptors in cells of the reproductive system.

Sperm cells were able to take up AEA through a carrier with the features of AMT (Fig. 3A). The molecular properties of AMT are not known and no probes are yet available to measure its expression (Hillard and Jarrahian, 2003). However, the observation that AMT in sperm cells has K_m and V_{max} values very close to those of human lymphocytes (Maccarrone et al., 2001), human endothelial cells (Maccarrone et al., 2002) and rat Sertoli cells (Maccarrone et al., 2003b), suggests that the same carrier may be present on the surface of different peripheral cells. Moreover, AEA uptake by sperm cells was completely blocked by VDM11, a selective AMT inhibitor (De Petrocellis et al., 2001). In addition, sperm cells had an active FAAH, whose affinity and maximum velocity towards AEA resembled those of the same enzyme in human lymphocytes (Maccarrone et al., 2003a) and in rat Sertoli cells (Maccarrone et al., 2003b). As in lymphocytes and Sertoli cells, AEA hydrolysis was fully blocked by MAFP, a selective FAAH inhibitor (De Petrocellis et al., 2001).

AEA is synthesized in sea urchin eggs (Bisogno et al., 1997) and in rodent testis, oviduct and uterus (Schuel et al., 2002a). It should be stressed that uterus contains the highest levels of AEA detected in mammalian organs, including brain (Paria and Dey, 2000). Here, we show evidence that boar sperm cells have the recently characterized AEA-synthesizing enzyme NAPE-PLD (Okamoto et al., 2004), and that they also contain endogenous AEA (Table 1). These findings, together with the assay of AEA in boar seminal plasma, extend recent data on the presence of AEA in reproductive fluids of mammals (Schuel et al., 2002a). This is the first reported evidence that sperm cells synthesize, transport and degrade AEA.

Altogether, it can be concluded that sperm are equipped with the typical machinery of cells regulated by endocannabinoids. In fact, following ejaculation they are exposed to these bioactive lipids, present both in seminal plasma and in the uterine tract (Schuel et al., 2002a; Schuel et al., 2002b). In this context, it is worth mentioning that, once spermatozoa are ejaculated into the female genital tract, two major functions must be accomplished in order to allow fertilization (reviewed in Yanagimachi, 1994). First, spermatozoa must undergo capacitation. This is a rather complex process that occurs physiologically in the female genital tract and consists essentially of acquiring the ability to recognise the egg (Cohen-Dayag and Eisnbach, 1994; de Lamirande et al., 1997). Alternatively, a complete capacitation can be induced in vitro by incubating the sperm in special media, that have been developed empirically to support in vitro fertilization (Cohen-Dayag et al., 1995). If capacitation is acquired, the second major function is accomplished as long as the spermatozoon encounters the egg, i.e. when specific receptors on the sperm membrane interact with the proteins of the ZP. In this event, only capacitated sperm activate the series of processes required for fertilization, amongst which acrosome reaction is the clue event. As capacitated sperm must undergo a quick and complete acrosome exocytosis upon interaction with the egg, the acrosome must be in a condition of high instability, which on the one hand allows immediate reactions, but on the other hand exposes the cell to the risk of premature or non-physiological AR. Indeed, spontaneous AR is an uncontrolled phenomenon of exocytosis that leads quickly to cell death (Yanagimachi, 1994). Once AR has occurred, sperm cell survival is markedly reduced, and more importantly their fertilizing ability is nearly exhausted (Harrison, 1996). Overall, capacitated sperm are in a rather unstable condition that combines their high reactive potential with the risk of non-targeted activation. The data presented in this paper demonstrate that endocannabinoids are involved in the regulation of these crucial phases that precede fertilization.

As far as capacitation is concerned, anandamide is inhibitory, and in fact sperm incubated under capacitating conditions in the presence of Met-AEA fail to undergo ZP-induced AR. In this respect, endocannabinoids resemble other regulatory factors identified in seminal plasma that inhibit the functional maturation of the sperm (Yanagimachi, 1994; Cross, 1996). Capacitation involves a series of functional and structural modifications of the cell, which are not yet fully determined. An increase in intracellular cAMP levels has been associated with capacitation, and indeed the ability of bicarbonate to stimulate capacitation in vitro has been related to activation of sperm adenylate cyclase (Chen et al., 2000). Our results strongly suggest that the inhibitory effect of Met-AEA on capacitation depends on its ability to reduce intracellular levels of cAMP (Table 2), a typical CB1R-mediated effect (Di Marzo et al., 2002; Howlett et al., 2002). The effects of the selective CB1R antagonist SR141716 and of the permeable analogue 8Br-cAMP (Fig. 6B) support the hypothesis that the activity of AEA on sperm capacitation is mediated by CB1R. Interestingly, these data give biochemical background to previous observations on human spermatozoa, whose motility is reduced by Met-AEA (Schuel et al., 2002), by Δ^9-tetrahydrocannabinol (Whan et al., 2004) or by AEA (Rossato et al., 2005). Incidentally, under our experimental conditions determination of motility was not reliable, because of the absence of BSA in the culture medium (Harrison et al., 1978; Harrison et al., 1982). However, it seems noteworthy that the present findings suggest that cAMP signaling, together with modulation of mitochondrial activity and membrane properties (Rossato et al., 2005), is a means to execute CB1R-dependent effects in sperm cells.

We also show that boar sperm cells express TRPV1 receptors, which are involved in the regulation of sperm function once capacitation has been acquired. While the binding to CB1R and TRPV1, and the activity of AMT, NAPE-PLD and (most markedly) FAAH are reduced after incubation under capacitating conditions, AEA levels in capacitated sperm were double that of the control levels (Table 1). This observation is supported by the partial reduction (64%) of the AEA-synthesizing NAPE-PLD and by the almost complete inhibition (9%) of the AEA-hydrolyzing FAAH in these cells (Table 1). Sperm are not able to synthesize proteins, therefore the quantitative reduction of CB1R, TRPV1 and FAAH upon capacitation (Fig. 2B) can be attributed to protein degradation. In keeping with this hypothesis, decrease of enzyme levels (Mack et al., 1983) and activation of proteases (Guerette et al.,

1988) in capacitated sperm have been already reported. The increased intracellular levels of AEA are likely to play a role in stabilizing the acrosome membrane of capacitated sperm, thus preventing non-targeted AR. In fact, when TRPV1 was blocked by the selective antagonist CPZ, a large proportion of spermatozoa incubated under capacitating conditions underwent AR without ZP stimulation, and the subsequent addition of ZP proteins could not further increase the extent of AR (Fig. 7). Interestingly, spontaneous exocytosis (i.e. that occurring in the absence of ZP stimulation) induced by CPZ could be prevented by co-administration of VDM11 (Fig. 7), a finding that is suggestive of a control by AMT. This observation can be explained by proposing that VDM11 inhibits AMT and hence AEA export from sperm (Maccarrone et al., 2002), as recently reported in neurons and other cell types (Ligresti et al., 2004). Consequently, intracellular AEA can displace CPZ from TRPV1 thus activating this receptor. The fact that treatment with VDM11 increased endogenous levels of AEA by approx. threefold favours this hypothesis. Overall, these data suggest that intracellular endocannabionoids can stabilize capacitated sperm by acting on TRPV1. Apparently such an activity becomes more effective with the progression of capacitation, that is paralleled by the increase of intracellular AEA (Table 1).

Taken together, sperm function seems to be regulated by endocannabinoids that exert a dual stage-dependent effect. However, AEA, present in both seminal plasma and uterine fluids, prevents premature capacitation in freshly ejaculated sperm via a CB1R-mediated mechanism. In this way, AEA contributes to maintaining a suitable environment for the sperm to travel along the uterine tract without any fertilizing potential, but in a condition of membrane stability. By contrast, a few hours later, when sperm have reached the oviduct (a condition that corresponds to the incubation in vitro under capacitating conditions), this inhibitory brake becomes less stringent. In fact, spermatozoa are exposed to a progressively reduced concentration of AEA in the proximal female genital tract (Shuel et al., 2002a), and sperm capacitation may occur as a consequence of CB1R disinhibition. At this time, intracellular endocannabinoids may become a major switch in the regulation of sperm function, and an increase in endogenous AEA may be necessary to activate TRPV1 and prevent spontaneous AR. As a consequence, AR will result only from sperm-egg interactions, thus maximizing the fertilizing potential of the sperm population. These endocannabinoid-mediated cross-talks between sperm and egg need further investigation. At any rate, the observation that sperm cells have a complete endocannabinoid system adds a new player to hormone/cytokine/endocannabinoid networks regulating fertility in mammals (Maccarrone and Finazzi-Agrò, 2004). In the same way, taking into account that exogenous cannabinoids lower testosterone secretion and impair sperm function (Hall and Solowij, 1998), the presence of the endocannabinoid system in spermatozoa suggests a possible physiological role of AEA in controlling male fertility. Overall, the present data provide new perspectives in the understanding and treatment of male fertility problems, and show that substances once thought to occur only in the female are also present in the male reproductive organs.

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References

Barboni, B., Mattioli, M. and Seren, E. (1995). Influence of progesterone on boar sperm capacitation. *J. Endocr.* **144**, 13-18.

Bisogno, T., Ventriglia, M., Milone, A., Mosca, M., Cimino, G. and Di Marzo, V. (1997). Occurrence and metabolism of anandamide and related acyl-ethanolamides in ovaries of the sea urchin Paracentrotus lividus. *Biochim. Biophys. Acta* **1345**, 338-348.

Bisogno, T., De, Petrocellis, L. and Di Marzo, V. (2002). Fatty acid amide hydrolase, an enzyme with many bioactive substrates. Possible therapeutic implications. *Curr. Pharm. Des.* **8**, 533-547.

Bisogno, T., Howell, F., Williams, G., Minassi, A., Cascio, M. G., Ligresti, A., Matias, I., Schiano-Moriello, A., Paul, P., Williams, E. J. et al. (2003). Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid knockout in the brain. *J. Cell Biol.* **163**, 463-468.

Bracey, M. H., Hanson, M. A., Masuda, K. R., Stevens, R. C. and Cravatt, B. F. (2002). Structural adaptations in a membrane enzyme that terminates endocannabinoid signaling. *Science* **298**, 1793-1796.

Chang, M. C., Berkery, D., Schuel, R., Laychock, S. G., Zimmerman, A. M., Zimmerman, S. and Schuel, H. (1993). Evidence for a cannabinoid receptor in sea urchin sperm and its role in blockade of the acrosome reaction. *Mol. Reprod. Dev.* **36**, 507-516.

Chen, Y., Cann, M. J., Litvin, T. N., Iourgenko, V, Sinclair, M. L., Levin, L. R. and Buck, J. (2000). Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. *Science* **289**, 625-628.

Cohen-Dayag, A. and Eisenbach, M. (1994). Potential assays for sperm capacitation in mammals. *Am. J. Physiol.* **267**, C1167-C1176.

Cohen-Dayag, A., Tur-Kaspa, I., Dor, J., Mashiach, S. and Eisenbach, M. (1995). Sperm capacitation in humans is transient and correlates with chemotactic responsiveness to follicular factors. *Proc. Natl. Acad. Sci. USA* **92**, 11039-11043.

Cross, N. L. (1996). Human seminal plasma prevents sperm from becoming acrosomally responsive to the agonist, progesterone: cholesterol is the major inhibitor. *Biol. Reprod.* **54**, 138-145.

Cross, N. L., Morales, P., Overstreet, J. H. and Hansono, F. W. (1986). Two simple methods for detecting acrosome-reacted human sperm. *Gamete Res.* **15**, 213-226.

Cross, N. L., Morales, P. and Behboodi, E. (1989). Determining acrosomal status of the cynomolgus monkey (Macaca fascicularis) sperm by fluorescence microscopy. *Am. J. Primatol.* **17**, 157-163.

de Lamirade, E., Leclerc, P. and Gagnon, C. (1997). Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. *Mol. Hum. Reprod.* **3**, 175-194.

De Petrocellis, L., Bisogno, T., Maccarrone, M., Davis, J. B., Finazzi-Agrò, A. and Di Marzo, V. (2001). The activity of anandamide at vanilloid VR1 receptors requires facilitated transport across the cell membrane and is limited by intracellular metabolism. *J. Biol. Chem.* **276**, 12856-12863.

De Petrocellis, L., Cascio, M. G. and Di Marzo, V. (2004). The endocannabinoid system: a general view and latest additions. *Br. J. Pharmacol.* **141**, 765-774.

Di Marzo, V., De Petrocellis, L., Fezza, F., Ligresti, A. and Bisogno, T. (2002). Anandamide receptors. *Prostag. Leukotr. Ess.* **66**, 377-391.

Dinh, T. P., Carpenter, D., Leslie, F. M., Freund, T. F., Katona, I., Sensi, S. L., Kathuria, S. and Piomelli, D. (2002). Brain monoglyceride lipase participating in endocannabinoid inactivation. *Proc. Natl. Acad. Sci. USA* **99**, 10819-10824.

Fride, E. (2002). Endocannabinoids in the central nervous system-an overview. *Prostag. Leukotr. Ess.* **66**, 221-233.

Fukami, K., Yoshida, M., Inoue, T., Kurokawa, M., Fissore, R. A., Yoshida, N., Mikoshiba, K. and Takenawa, T. (2003). Phospholipase Cδ4 is required for Ca^{2+} mobilization essential for acrosome reaction in sperm. *J. Cell Biol.* **1**, 79-88.

Gadella, B. M. and Harrison, R. A. (2000). The capacitating agent bicarbonate induces protein kinase A-dependent changes in phospholipid transbilayer behavior in the sperm plasma membrane. *Development* **127**, 2407-2420.

Gadella, B. M., Lopes-Cardozo, M., van Golde, L. M., Colenbrander, B.

and Gadella, T. W, Jr (1995). Glycolipid migration from the apical to the equatorial subdomains of the sperm head plasma membrane precedes the acrosome reaction. Evidence for a primary capacitation event in boar spermatozoa. *J. Cell Sci.* **108**, 935-946.

Gerard, C. M., Mollereau, C., Vassart, G. and Parmentier, M. (1991). Molecular cloning of a human cannabinoid receptor which is also expressed in testis. *Biochem. J.* **279**, 129-134.

Guerette, P., Langlais, J., Antaki, P., Chapdelaine, A. and Roberts, K. D. (1988). Activation of phospholipase A_2 of human spermatozoa by proteases. *Gamete Res.* **19**, 203-214.

Hall, W. and Solowij, N. (1998). Adverse effects of cannabis. *Lancet* **352**, 1611-1616.

Harrison, R. A. (1996). Bicarbonate/CO2, an effector of capacitation, induces a rapid and reversible chenge in the lipid architecture of boar sperm plasma membranes *Mol. Reprod. Dev.* **45**, 378-391.

Harrison, R. A. (1997). Sperm plasma membrane characteristics and boar semen fertility. *J. Reprod. Fertil.* **52**, S195-S211.

Harrison, R. A. (2004). Rapid PKA-catalysed phosphorilation of boar sperm proteins induced by the capacitating agent bicarbonate. *Mol. Reprod. Dev.* **67**, 337-352.

Harrison, R. A., Dott, H. M. and Foster, G. C. (1978). Effect of ionic strength, serum albumin and other macromolecules on the maintenance of motility and the surface of mammalian spermatozoa in a simple medium. *J. Reprod. Fertil.* **52**, 65-73.

Harrison, R. A., Dott, H. M. and Foster, G. C. (1982). Bovine serum albumin, sperm motility, and the "dilution effect". *J. Exp. Zool.* **222**, 81-88.

Hillard, C. J. and Jarrahian, A. (2003). Cellular accumulation of anandamide: consensus and controversy. *Br. J. Pharmacol.* **140**, 802-808.

Howlett, A. C., Barth, F., Bonner, T. I., Cabral, G., Casellas, P., Devane, W. A., Felder, C. C., Herkenham, M., Mackie, K., Martin, B. R. et al. (2002). International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol. Rev.* **54**, 161-202.

James, P. S., Hennessy, C., Berge, T. and Jones, R. (2004). Compartmentalisation of the sperm plasma membrane: a FRAP, FLIP and SPFI analysis of putative diffusion barriers on the sperm head. *J. Cell Sci.* **117**, 6485-6495.

Jordt, S. E. and Julius, D. (2002). Molecular basis for species-specific sensitivity to "hot" chili peppers. *Cell* **108**, 421-430.

Ligresti, A., Morera, E., Van Der Stelt, M., Monory, K., Lutz, B., Ortar, G. and Di Marzo, V. (2004). Further evidence for the existence of a specific process for the membrane transport of anandamide. *Biochem. J.* **380**, 265-272.

Lin, S., Khanolkar, A. D., Fan, P., Goutopoulos, A., Qin, C., Papahadjis, D. and Makriyannis, A. (1998). Novel analogues of arachidonylethanolamide (anandamide): affinities for the CB1 and CB2 receptors and metabolic stability. *J. Med. Chem.* **41**, 5353-5361.

Logan, J. S. and Sharma, A. (1999). Potential use of genetically modified pigs as organ donors for transplantation into humans. *Clin. Exp. Pharmacol. Physiol.* **26**, 1020-1025.

Maccarrone, M. and Finazzi-Agrò, A. (2004). Anandamide hydrolase: a guardian angel of human reproduction? *Trends Pharmacol. Sci.* **25**, 353-357.

Maccarrone, M., Lorenzon, T., Bari, M., Melino, G. and Finazzi-Agrò, A. (2000). Anandamide induces apoptosis in human cells *via* vanilloid receptors. Evidence for a protective role of cannabinoid receptors. *J. Biol. Chem.* **275**, 31938-31945.

Maccarrone, M., Valensise, H., Bari, M., Lazzarin, N., Romanini, C. and Finazzi-Agrò, A. (2001). Progesterone up-regulates anandamide hydrolase in human lymphocytes. Role of cytokines and implications for fertility. *J. Immunol.* **166**, 7183-7189.

Maccarrone, M., Bari, M., Battista, N. and Finazzi-Agrò, A. (2002). Estrogen stimulates arachidonoylethanolamide release from human endothelial cells and platelet activation. *Blood* **100**, 4040-4048.

Maccarrone, M., Bari, M., Di Rienzo, M., Finazzi-Agrò, A. and Rossi, A. (2003a). Progesterone activates fatty acid amide hydrolase (FAAH) promoter in human T lymphocytes through the transcription factor Ikaros. Evidence for a synergistic effect of leptin. *J. Biol. Chem.* **278**, 32726-32732.

Maccarrone, M., Cecconi, S., Rossi, G., Battista, N., Pauselli, R. and Finazzi-Agrò, A. (2003b). Anandamide activity and degradation are regulated by early postnatal ageing and follicle-stimulating hormone in mouse Sertoli cells. *Endocrinology* **144**, 20-28.

Mack, S., Bhattacharyya, A. K., Joyce, C., Van der Ven, H. and Zaneveld, L. J. (1983). Acrosomal enzymes of human spermatozoa before and after in vitro capacitation. *Biol. Reprod.* **28**, 1032-1042.

Maxwell, W. M. C. and Johnson, L. A. (1997). Chlortetracycline analysis of boar spermatozoa after incubation, flow cytometric sorting, cooling or cryopreservation. *Mol. Repr. Dev.* **46**, 408-413.

Mattioli, M., Barboni, B., Lucidi, P. and Seren, E. (1996). Identification of capacitation in boar spermatozoa by chlortetracycline staining. *Theriogenology* **45**, 373-377.

Mechoulam, R. (2002). Discovery of endocannabinoids and some random thoughts on their possible roles in neuroprotection and aggression. *Prostag. Leukotr. Ess.* **66**, 93-99.

Murphy, L. L., Gher, J., Steger, R. W. and Bartke, A. (1994). Effects of delta 9-tetrahydrocannabinol on copulatory behavior and neuroendocrine responses of male rats to female conspecifics. *Pharmacol. Biochem. Behav.* **48**, 1011-1017.

Oddi, S., Bari, M., Battista, N., Barsacchi, D., Cozzani, I. and Maccarrone, M. (2005). Confocal microscopy and biochemical analysis reveal spatial and functional separation between anandamide uptake and hydrolysis in human keratinocytes. *Cell. Mol. Life Sci.* **62**, 386-395.

Okamoto, Y., Morishita, J., Tsuboi, K., Tonai, T. and Ueda, N. (2004). Molecular characterization of a phospholipase D generating anandamide and its congeners. *J. Biol. Chem.* **279**, 5298-5305.

Palmer, S. L., Thakur, G. A. and Makriyannis, A. (2002). Cannabinergic ligands. *Chem. Phys. Lipids* **121**, 3-19.

Paria, B. C. and Dey, S. K. (2000). Ligand-receptor signaling with endocannabinoids in preimplantation embryo development and implantation. *Chem. Phys. Lipids* **108**, 211-220.

Park, B., Gibbons, H. M., Mitchell, M. D. and Glass, M. (2003). Identification of the CB1 cannabinoid receptor and fatty acid amide hydrolase (FAAH) in the human placenta. *Placenta* **24**, 990-995.

Park, B., McPartland, J. M. and Glass, M. (2004). Cannabis, cannabinoids and reproduction. *Prostag. Leukotr. Ess.* **70**, 189-197.

Parolaro, D., Massi, P., Rubino, T. and Monti, E. (2002). Endocannabinoids in the immune system and cancer. *Prostag. Leukotr. Ess.* **66**, 319-332.

Pertwee, R. G. (1997). Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacol. Ther.* **74**, 129-180.

Ralevic, V., Kendall, D. A., Jerman, J. C., Middlemiss, D. N. and Smart, D. (2001). Cannabinoid activation of recombinant and endogenous vanilloid receptors. *Eur. J. Pharmacol.* **424**, 211-219.

Ross, R. A., Gibson, T. M., Brockie, H. C., Leslie, M., Pashmi, G., Craib, S. J., Di Marzo, V. and Pertwee, R. G. (2001). Structure-activity relationship for the endogenous cannabinoid, anandamide, and certain of its analogues at vanilloid receptors in transfected cells and vas deferens. *Br. J. Pharmacol.* **132**, 631-640.

Rossato, M., Popa, F. I., Ferigo, M., Clari, G. and Foresta, C. (2005). human sperm express cannabinoid receptor CB1 whitc activation inhibits motility, acrosome reaction and mitochondrial function *J. Clin. Endocrinol. Metabol.* **90**, 984-991.

Schuel, H., Goldstein, E., Mechoulam, R., Zimmerman, A. M. and Zimmerman, S. (1994). Anandamide (arachidonylethanolamide), a brain cannabinoid receptor agonist, reduces sperm fertilizing capacity in sea urchins by inhibiting the acrosome reaction. *Proc. Natl. Acad. Sci. USA* **91**, 7678-7682.

Schuel, H., Burkman, L. J., Lippes, J., Crickard, K., Forester, E., Piomelli, D. and Giuffrida, A. (2002a). N-Acylethanolamines in human reproductive fluids. *Chem. Phys. Lipids* **121**, 211-227.

Schuel, H., Burkman, L. J., Lippes, J., Crickard, K., Mahony, M. C., Giuffrida, A., Picone, R. P. and Makriyannis, A. (2002b). Evidence that anandamide-signaling regulates human sperm functions required for fertilization. *Mol. Reprod. Dev.* **63**, 376-387.

Suarez, S. S. and Ho, H. C. (2003). Hyperactivation of mammalian sperm. *Cell. Mol. Biol.* **49**, 351-356.

Sugiura, T., Kondo, S., Sukagawa, A., Tonegawa, T., Nakane, S., Yamashita, A. and Waku, K. (1996). Enzymatic synthesis of anandamide an endogenous cannabinoid receptor ligand through N-acylphosphatidylethanolamine pathway in testis: involvement of Ca^{2+}-dependent transacylase and phosphodiesterase activities. *Biochem. Biophys. Res. Commun.* **218**, 113-117.

Van der Stelt, M. and Di Marzo, V. (2004). Endovanilloids. Putative endogenous ligands of transient receptor potential vanilloid 1 channels. *Eur. J. Biochem.* **271**, 1827-1834.

Ward, C. R. and Storey, B. T. (1984). Determination of the time course of capacitation in mouse spermatozoa using a chlortetracycline fluorescence assay. *Dev. Biol.* **104**, 287-296.

Wang, H., Matsumoto, H., Guo, Y., Paria, B. C., Roberts, R. L. and Dey, S. K. (2003). Differential G protein-coupled cannabinoid receptor knockout by anandamide directs blastocyst activation for implantation. *Proc. Natl. Acad. Sci. USA* **100**, 14914-14919.

Wang, W. H., Abeydeera, L. R., Fraser, L. R. and Niwa, K. (1995). Functional analysis using chlortatracycline flurescence and in vitro fertilization of frozen-thawed ejaculated boar spermatozoa incubated in a protein-free chemically defined medium *J. Reprod. Fert.* **104**, 303-315.

Wang, Y., Liu, Y., Ito, Y., Hashiguchi, T., Kitajama, I., Yamakuchi, M., Shimizu, H., Matsuo, S., Imaizumi, H. and Maruyama, I. (2001). Simultaneous measurement of anandamide and 2-arachidonoylglycerol by polymixin B-selective adsorption and subsequent high-performance liquid chromatography analysis: increase in endogenous cannabinoids in the sera of patients with endotoxic shock. *Anal. Biochem.* **294**, 73-82.

Wenger, T., Ledent, C., Csernus, V. and Gerendai, I. (2001). The central cannabinoid receptor inactivation suppresses endocrine reproductive functions. *Biochem. Biophys. Res. Commun.* **284**, 363-368.

Wennemuth, G., Babcock, D. F. and Hille, B. (2003). Calcium clearance mechanisms of mouse sperm. *J. Gen. Physiol.* **122**, 115-128.

Whan, L. B., McClure, N. and Lewis, S. E. M. (2004). The effects of tetrahydrocannabinol (THC), the primary psychoactive cannabinoid in marijuana, on in vitro human sperm motility. Proceedings of the 14th Annual Symposium on the Cannabinoids (22-27 June 2004, Paestum, Italy), p. 178.

Yanagimachi, R. (1994). Mammalian fertilization In *The Physiology of Reproduction*. 2nd edn (ed. E. Knobil and J. D. Neill), pp, 189-196. New York: Raven Press.

Zygmunt, P. M., Petersson, J., Andersson, D. A., Chuang, H.-H., Sorgård, M., Di Marzo, V., Julius, D. and Högestätt, E. D. (1999). Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* **400**, 452-457.