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## Effect of capacitation on the endocannabinoid system of mouse sperm

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**ABSTRACT**

The presence of the elements of the endocannabinoid system (ECS) in sperm isolated from several species (from invertebrates to mammals, humans included) has supported the “evolutionary theory” that proposes endocannabinoids as check points in reproductive events like capacitation. In this study, we characterized the ECS elements at the mRNA, protein and functional levels in mouse sperm before and after capacitation. We found that the latter process increases the endogenous levels of the two major endocannabinoids (anandamide and 2-arachidonoylglycerol), through a decreased degradation and increased biosynthesis, respectively. Additionally, we found that the binding activity of cannabinoid receptors was not affected by sperm capacitation, whereas that of vanilloid receptor was reduced. Overall, our data demonstrate that mouse sperm have a fully functional ECS, and that capacitation alters the endogenous tone of the major endocannabinoids through distinct mechanisms.

## INTRODUCTION

Endocannabinoids (eCBs) are lipid compounds able to bind to both type 1 (CB<sub>1</sub>) and type 2 (CB<sub>2</sub>) cannabinoid receptors (Howlett *et al.*, 2002). In the last few years a great deal of data have been accumulated, suggesting that eCBs have important actions in the reproductive system. In fact, all the essential components of the ECS have been demonstrated in sperm from various mammals (reviewed by Battista *et al.*, 2008; Pierantoni *et al.*, 2009). The presence of CB<sub>1</sub>, but not CB<sub>2</sub>, in both testicular and isolated sperm from all analyzed vertebrates suggests an evolutionarily conserved role of CB<sub>1</sub>-dependent eCBs signalling in sperm physiology (Pierantoni *et al.*, 2009). In fact AEA, the best characterized eCB together with 2-arachidonoylglycerol (2-AG), is implicated in the inhibition of sperm motility and acrosome reaction via CB<sub>1</sub> activation (Maccarrone *et al.*, 2005; Rossato *et al.*, 2005; Cobellis *et al.*, 2006), and inhibits sperm capacitation, a complex series of structural and functional changes which include reorganization of sperm membrane phospholipids and proteins (Gadella *et al.*, 2008), reduction in membrane cholesterol levels (Yanagimachi, 1994), and changes in sperm motility, known as hyperactivation (Yanagimachi, 1994). Moreover, AEA has also been detected in female reproductive fluids (Schuel *et al.*, 2002), where capacitation normally occurs, further supporting the idea that eCBs are check-points in reproduction. More recently, a key role for 2-AG has been proposed in the modulation of mouse spermatogenesis (Grimaldi *et al.*, 2009). However, the molecular details of ECS elements and eCBs signalling in controlling sperm function and male fertility remain elusive (Maccarrone, 2009; Taylor *et al.*, 2010). The aim of this study was to ascertain the presence and functionality of the main ECS elements in mouse sperm, and the possible effect of capacitation on their activity and expression. To this end, we measured in mouse sperm mRNA, protein and activity of the AEA synthetase NAPE-PLD (*N*-acylphosphatidylethanolamine-specific phospholipase D) and hydrolase FAAH (fatty acid amide hydrolase), of the 2-AG synthetase DAGL (diacylglycerol lipase) and hydrolase MAGL (monoacylglycerol lipase), and of their CB<sub>1</sub> and CB<sub>2</sub> target receptors (Bari *et al.*, 2010). In addition, we ascertained the presence of the AEA-binding TRPV1 (transient receptor potential vanilloid 1) channels, that control the acrosome reaction (Maccarrone *et al.*, 2005) and modulate sperm-oocyte interactions in humans (Francavilla *et al.*, 2009).

## MATERIALS and METHODS

Chemicals were of the purest analytical grade. Anandamide (*N*-arachidonoyl ethanolamine, AEA) and 5-(1,10-dimethylheptyl)-2-[(*1R,5R*)-hydroxy-(*2R*)-(3-hydroxypropyl)-cyclohexyl] phenol (CP55940) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 2-Arachidonoylglycerol (2-AG) and resiniferatoxin (RTX) were from Alexis (San Diego, CA, USA). AEA-ethanolamine-1- $^3\text{H}$  (60 Ci/mmol),  $^3\text{H}$ CP55.940 (126 Ci/mmol) and  $^3\text{H}$ RTX (43 Ci/mmol) were purchased from Perkin Elmer Life Sciences (Boston, MA, USA). *N*- $^3\text{H}$ Arachidonoyl-phosphatidylethanolamine ( $^3\text{H}$ NArPE, 200 Ci/mmol) was from ARC (St. Louis, MO, USA).  $^{14}\text{C}$ 1-Stearoyl-2-arachidonoyl-*sn*-glycerol (56 mCi/mmol) was from Amersham Biosciences.  $\text{d}_8$ -AEA,  $\text{d}_8$ -2-AG, rabbit anti-NAPE-PLD and anti-MAGL polyclonal antibodies were from Cayman Chemicals (Ann Arbor, MI, USA). Rabbit anti-CB<sub>1</sub> and anti-CB<sub>2</sub> polyclonal antibodies were from ABR-Affinity BioReagents (Golden, CO, USA); rabbit anti-TRPV1 and anti-FAAH polyclonal antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA); rabbit anti-DAGL polyclonal antibodies were from Frontier Science Co. Ltd (Okkaido, Japan) Goat Alexa Fluor conjugates secondary antibodies and Prolong antifade kit were from Molecular Probes (Eugene, OR, USA).

### Animals

Adult (90- to 120-day-old) swiss CD1 male mice of proven fertility, and 2-month-old swiss CD1 female mice were purchased from Charles River Italia (Como, Italy). All efforts were made to minimize animal suffering and to reduce the number of mice used, in accordance with the European Communities Council Directive of 24 November, 1986 (86/609/EEC). The local committees on animal care and use approved all experimental protocols, that were applied according to accepted veterinary medical practice.

### Sperm preparation

Spermatozoa were released from the cauda epididymis by mincing the large diameter tubules in 1 ml of warmed equilibrated HTF medium, supplemented with 0.4% BSA to obtain capacitated sperm, or as such for resting sperm, respectively. After removing tissue fragments, sperm were capacitated for 2 h at 37°C. Both capacitated and resting spermatozoa were washed, counted and frozen.

### *In vitro* fertilization

*In vitro* fertilization (IVF) was performed on ovulated eggs (n= 150/group), which were devoid of zona pellucida. Eggs were inseminated for 1 h and analyzed for the presence of male and female pronuclei 6 h later, as previously described (n = 150) (Cecconi *et al.*, 1996).

### **Cholesterol quantitation**

Sperm were homogenized and membrane lipids were extracted, evaporated under N<sub>2</sub> stream and fractionated on silica polypropylene plates by thin layer chromatography, as reported (Maccarrone *et al.*, 2009). Then, plates were dried and placed on a Heidolph plate heater, cholesterol bands were scanned using an Image Scanner (Amersham Biosciences, Fairfield, CT, USA), and the values obtained were quantitated by means of standard curves obtained with cholesterol standards.

### **qRT-PCR analysis**

RNA was extracted from sperm using the RNeasy extraction kit (Qiagen, Crawley, UK), as suggested by the manufacturer. Quantitative real time reverse transcriptase-polymerase chain reaction (qRT-PCR) assays were performed as previously described (Grimaldi *et al.*, 2009).

### **Endogenous levels of AEA and 2-AG**

Sperm were subjected to lipid extraction with chloroform/methanol (2:1, v/v). Endocannabinoid content was determined by liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS), as described (Francavilla *et al.*, 2009).

### **Receptor binding assays**

Cannabinoid and vanilloid receptor studies were performed by rapid filtration assays, as previously reported (Francavilla *et al.*, 2009).

### **Enzymatic activities**

The metabolism of AEA through NAPE-PLD and FAAH, and that of 2-AG through DAGL and MAGL were assayed as detailed elsewhere (Catani *et al.*, 2009).

### **Immunocytochemical analysis**

Immunocytochemistry was performed as detailed elsewhere (Catanzaro *et al.*, 2009), using the following antibody dilutions: anti-CB<sub>1</sub> (1:400), anti-CB<sub>2</sub> (1:400), anti-TRPV1 (1:100), anti-NAPE-PLD (1:400), anti-FAAH (1:200), anti-DAGL (1:200) or anti-MAGL (1:400). Pictures were taken using the LAS AF program (Leica Microsystems). Quantification was performed using 5 images for each condition, and measuring fluorescence intensity of each sperm cell in the field (10 cells were analyzed for each image). Non-specific fluorescence was always subtracted from the total fluorescence (see Supplementary Material for negative controls). Also bright-field images were taken from resting and capacitated sperm, in order to check the anatomical features of these cells (Supplementary Material).

### Statistical analysis

Statistical analysis was performed through paired Student's *t* test for IVF data, and unpaired Student's *t* test for the other experiments (GraphPAD Software for Science, San Diego, CA, USA). All data were reported as mean  $\pm$  S.E.M. of at least three independent experiments, each performed in duplicate.

## RESULTS

### *In vitro* fertilization and capacitation of mouse sperm

Metaphase II-arrested oocytes were incubated with resting and capacitated mouse sperm and their ability to form a male pronucleus was tested. As reported in Table 1, 70% of oocytes showed the presence of female and male pronuclei following IVF with capacitated mouse sperm, compared to 3% of those fertilized with resting sperm. In addition, reduction in membrane cholesterol content (down to ~45%), an hallmark of capacitation (Go and Wolf, 1985; Belmonte *et al.*, 2005; Gadella *et al.*, 2008), was documented in capacitated *versus* resting sperm (Table 2), further corroborating that the capacitation process was fully effective.

### Presence of active ECS elements in mouse sperm

Resting and capacitated mouse sperm express all major ECS components both at mRNA (Fig. 1) and protein (Fig. 2) levels. All ECS elements were functional, as demonstrated through activity and binding assays (Table 3). In particular, NAPE-PLD activity was not affected by the capacitation process, whereas FAAH activity significantly decreased by ~25% (Table 3). Conversely, capacitation dramatically increased DAGL activity (~10-fold over resting cells), while slightly reducing MAGL activity (Table 3). In keeping with these activity data, both endogenous levels of AEA and 2-AG increased in capacitated *versus* resting sperm, up to ~9-fold and ~4-fold respectively (Table 2). Incidentally, in the case of AEA these data corroborate previous findings in boar sperm (Maccarrone *et al.*, 2005). In addition, the binding activity of CB receptors was not affected by capacitation, whereas that of TRPV1 was reduced by ~20% (Table 3). These functional data were paralleled by alterations of gene expression at the transcriptional and translational levels, that were consistent with the activity data for MAGL (with a non-significant trend for FAAH, DAGL and TRPV1) at the mRNA level (Fig. 1), and for DAGL, CBR and TRPV1 (with a non-significant trend for MAGL) at the protein level (Fig. 2). The data on protein expression of DAGL and TRPV1 were further corroborated by Western blotting and enzyme-linked immunosorbent assay (ELISA), shown in the Supplementary Material. It can be noted that mRNA and protein expression of some of the ECS elements (e.g., CB<sub>1</sub>R and FAAH) were differently affected by capacitation. In this context, there are several reports showing significant differences between transcript and protein levels of ECS components under different experimental paradigms (Fonseca

*et al.*, 2009; Bari *et al.*, 2011). These reports and our data suggest that the expression of ECS genes can be regulated in a different manner at the transcription and translation levels.

### **Localization of ECS elements in mouse sperm**

The localization of ECS components in resting and capacitated mouse sperm was determined also by means of confocal microscopy. CB<sub>1</sub> was found to be restricted in the anterior and post-acrosomal region of sperm head, whereas CB<sub>2</sub> was localized at the sperm head in the post-acrosomal region and, less intensely, in the sperm tail; only in a few cells, CB<sub>2</sub> staining was observed in the front part of the head (Fig. 2). The other ECS elements were clearly detectable on the sperm head, where they seemed to be confined in the post-acrosomal region (Fig. 2). Moreover, NAPE-PLD was detected on the sperm tail, and FAAH was detected on the whole sperm cell. The subcellular localization of all ECS members did not change in capacitated *versus* resting sperm (Fig. 2).

### **DISCUSSION**

In this study we demonstrate that mouse sperm, much alike boar (Maccarrone *et al.*, 2005) and human sperm (Francavilla *et al.*, 2009), express the major components of ECS needed to synthesize and degrade the two most prominent eCBs, AEA and 2-AG. We also show that mouse sperm express functional eCBs-binding CB<sub>1</sub>, CB<sub>2</sub> and TRPV1 receptors. It should be noted that in mouse sperm protein expression of CB<sub>2</sub> was almost the same as that of CB<sub>1</sub> (Fig. 2), at variance with its faint expression reported in boar (Maccarrone *et al.*, 2005) and human sperm (Francavilla *et al.*, 2009). In line with this, a recent study has shown CB<sub>2</sub> in the developing mouse sperm, disclosing a pivotal role for this CB receptor subtype in spermatogenesis (Grimaldi *et al.*, 2009). However, neither CB<sub>1</sub> nor CB<sub>2</sub> were affected by the capacitation process, whereas TRPV1, that binds AEA but not 2-AG, was clearly down-regulated. These data suggest that TRPV1-mediated activities of AEA can be critical also for mouse sperm, and the increase of the intracellular levels of AEA in capacitated sperm (Table 2), due to reduced FAAH activity (Table 3), further supports this hypothesis. On the other hand, only recently a few studies have focused on the role of 2-AG in male fertility, disclosing its impact on mouse spermatogenesis (Grimaldi *et al.*, 2009) and epididymal start-up (Cobellis *et al.*, 2010). Interestingly, in those studies the metabolic control of 2-AG content was shown to be due to enhanced transcriptional and translational levels of DAGL, and decreased expression of MAGL (Grimaldi *et al.*, 2009). Here, we show that capacitation appears to recapitulate this type of regulation, because it increases 2-AG content by enhancing the activity and expression of DAGL, while reducing that of MAGL. In this context, it seems noteworthy that the capacitation process leads to a hyperactive tone of both AEA and 2-AG, although through distinct mechanisms: reduced degradation by FAAH (in the case of AEA), or enhanced synthesis by DAGL (in the case of 2-AG). It can be suggested that these distinct pathways might allow a differential regulation of the content, and hence of the biological activity, of the two major eCBs, an

unprecedented observation that might extend well-beyond reproductive events. It should be recalled that in previous studies we have shown that intracellular levels of AEA increase during capacitation and contribute to promote the fertilizing ability of both boar (Maccarrone *et al.*, 2005) and human sperm (Francavilla *et al.*, 2009), by activating TRPV1 receptors at an intracellular binding site. In addition, AEA present in both seminal plasma and uterine fluids prevents premature capacitation in freshly ejaculated sperm via a CB<sub>1</sub>R-mediated mechanism (Battista *et al.*, 2008). Overall, it can be speculated that an increase of intracellular AEA can activate TRPV1 receptors and, by means of an export mechanism through a membrane transporter, it can contribute to maintain a suitable extracellular concentration for the activation of CB<sub>1</sub>R, thus facilitating the sperm journey along the uterine tract. A further activation of CB<sub>1</sub>R can be warranted also by intracellular 2-AG, once exported outside the cell. Therefore the two eCBs, by acting either extracellularly at CB<sub>1</sub>R or intracellularly at TRPV1, may play a key-role in controlling capacitation, as well as the spatio-temporal interaction of sperm with egg and the sperm-oocyte fusion process.

In conclusion, we show that mouse sperm have a fully functional endocannabinoid system, able to metabolize and bind the two major eCBs: AEA and 2-AG. We also show that capacitation remarkably enhances the endogenous tone of both eCBs through distinct mechanisms, a finding that might open new opportunities for therapeutic intervention. The present data might also help to develop transgenic mice, where different ECS elements can be knocked down or knocked in, and then can be exploited to further understand dysfunctions in male fertility.

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## Figure Legends

**Figure 1. qRT-PCR analysis of ECS elements in mouse sperm.** Representative quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) analysis of mRNA expression of ECS members in resting and capacitated mouse sperm. The mRNA level of each element of the ECS was expressed as fold over NAPE-PLD mRNA, set to 1.0 for resting sperm. \*Denotes  $p < 0.05$  versus resting sperm.

**Figure 2. Immunocytochemical analysis of ECS in mouse sperm.** The presence and localization of endocannabinoid-binding  $CB_1$ ,  $CB_2$  and TRPV1 receptors, of AEA metabolic enzymes (NAPE-PLD and FAAH), and of 2-AG metabolic enzymes (DAGL and MAGL) were ascertained in resting and capacitated sperm by means of confocal laser-scanning microscopy. Non-specific immunofluorescence in these cells was negligible for all tested antibodies (Supplementary material). The y-axis values represent the mean fluorescence per sperm. \*Denotes  $p < 0.05$  and \*\*  $p < 0.01$  versus resting sperm. Images were taken using a confocal microscope equipped with a 63X objective (numerical aperture = 1.4), and are representative of at least three independent experiments; five fields were examined for each treatment. Bars, 10  $\mu\text{m}$ .

**Table 1. Percentage of oocytes showing two pronuclei after IVF with resting or capacitated mouse sperm.**

<b>Sperm condition</b>	<b>Total number of inseminated eggs</b>	<b>Number of eggs with two pronuclei</b>
Resting	150	5 ± 2
Capacitated	150	105 ± 6 *

\*Denotes  $p < 0.01$  versus eggs inseminated with resting sperm.

**Table 2. Membrane cholesterol content, and endogenous levels of AEA and 2-AG in resting and capacitated mouse sperm.**

Parameter	Resting	Capacitated
Cholesterol content <sup>a</sup>	21.9 ± 0.9	10.0 ± 2.5*
Endogenous levels of AEA <sup>b</sup>	0.3 ± 0.1	2.7 ± 0.3**
Endogenous levels of 2-AG <sup>b</sup>	9.6 ± 1.1	38.3 ± 9.9*

<sup>a</sup>Expressed as µg/mg of protein.

<sup>b</sup>Expressed as fmol/mg of protein.

\*Denotes p<0.05 and \*\* p<0.01 *versus* resting sperm.

**Table 3. Activity of ECS elements in resting and capacitated mouse sperm.**

Parameter	Resting	Capacitated
NAPE-PLD activity <sup>a</sup>	13.1 ± 2.8	9.2 ± 1.7
FAAH activity <sup>a</sup>	175 ± 3	133 ± 11*
DAGL activity <sup>a</sup>	57 ± 6	581 ± 58**
MAGL activity <sup>a</sup>	1596 ± 4	1496 ± 8*
CBRs binding <sup>b</sup>	23.3 ± 0.9	24.0 ± 0.6
TRPV1 binding <sup>b</sup>	64.8 ± 1.1	53.3 ± 2.5*

<sup>a</sup>Expressed as pmol/min per mg of protein.

<sup>b</sup>Expressed as fmol/mg of protein.

\*Denotes p<0.05 and \*\*p<0.01 *versus* resting sperm.

### Research Highlights

- 1) Characterization of the endocannabinoid system (ECS) in mouse sperm;
- 2) Effects of capacitation on the expression of ECS elements;
- 3) Differential upregulation of endocannabinoid tone AEA and 2-AG during capacitation.

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