Is there a role for endocannabinoids in sperm–oviduct interaction?

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Abstract

The endocannabinoid system (ECS) has been found in reproductive cells and tissues in several mammals. Spermatozoa are able to respond to anandamide, and the oviduct is able to synthesize and modulate the concentration of this endocannabinoid along the isthmic and ampullary regions. The main aim of this study was to understand whether the ECS has a role during sperm storage and release within the oviduct in cattle. Data showed that 1) the endocannabinoid receptors 1 and 2 (CB1 and CB2) are present in bovine spermatozoa both in the initial ejaculate and in spermatozoa bound to the oviduct *in vitro*; 2) CB1 receptor is still detectable in spermatozoa released from the oviduct through penicillamine but not in those released through heparin; 3) arachidonylethanolamide (AEA) does not affect sperm viability, whereas it depresses sperm progressive motility and kinetic values; 4) sperm–oviduct binding and release *in vitro* are not influenced by AEA; 5) AEA depresses sperm–zona pellucida (ZP) binding; 6) binding of heparin-capacitated spermatozoa to the ZP is not affected by AEA; 7) *N*-acylphosphatidylethanolamine-selective phospholipase D, the main enzyme involved in anandamide synthesis, is expressed in oviductal epithelial cells. In conclusion, secretion of AEA from epithelial cells might contribute to the oviduct sperm-reservoir function, prolonging the sperm fertile life through the depression of motility and capacitation. Capacitation signals, such as heparin, that promote sperm release, might remodel the sperm surface and cause a loss of the sperm sensitivity to AEA.

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Introduction

Research on the chemical and pharmacological mechanisms responsible for psychoactive properties of tetrahydrocannabinol in marijuana led to the discovery that several animals can synthesize a group of related unsaturated fatty acid derivatives named endocannabinoids, which act as endogenous ligands for the cannabinoid receptors. Arachidonylethanolamide (anandamide, AEA), the first endogenous agonist for cannabinoid receptors, and 2-arachidonoyl glycerol are the main endocannabinoids within biological fluids (Devane *et al.* 1992, Felder & Glass 1998). In several tissues, AEA is released 'on demand' from membrane phospholipid precursors by *N*-acylphosphatidylethanolamine-selective phospholipase D (NAPEPLD) in response to cell depolarization or mobilization of intracellular Ca²⁺ stores. Released AEA is quickly degraded by membrane-bound fatty acid amide hydrolase (FAAH; Habayeb *et al.* 2002).

AEA regulates several functions in different tissues, via interaction with two specific G protein-coupled receptors, cannabinoid receptor 1 (CB1), and

cannabinoid receptor 2 (CB2), whose tissue distribution and function have been described in different animal models (McPartland *et al.* 2007). It is known that CB1, CB2, NAPEPLD, and FAAH are widely distributed in non-neuronal somatic cells of peripheral organs, and have been found in mammalian reproductive organs, as well as in germ cells of vertebrates and invertebrates (Habayeb *et al.* 2002). A possible role of AEA in reproduction is suggested by its high level in reproductive secretions (Schuel *et al.* 2002a, 2002b). In the male, CB1 is expressed in germ cells of both invertebrates and mammals, from spermatogonia to mature spermatozoa (Cobellis *et al.* 2006). A finely tuned microenvironment operated by AEA levels negatively influences the spermatogenetic process, reducing germ cell proliferation and reproductive organ weight (Cacciola *et al.* 2008, Lewis & Maccarrone 2009). Apoptosis of Sertoli cells, which nurse germinal cells during their maturation inside the seminiferous tubule, is mediated through a CB2-dependent mechanism (Maccarrone *et al.* 2003). Recently, AEA has been shown to depress motility and capacitation of human spermatozoa (Rossato *et al.* 2005). Moreover, boar spermatozoa are endowed with the

biochemical machinery to synthesize, bind, and degrade AEA (Maccarrone *et al.* 2005). Endocannabinoid signaling via CB1 seems to be involved during mouse embryo cleavage and transport in the oviduct (Wang *et al.* 2004). A role of embryonic FAAH in reducing AEA levels at potential implantation sites in the mouse is suggested by the expression of NAPEPLD from fertilization through the blastocyst stage and upregulation of FAAH in the morula and in blastocyst trophoctoderm (Wang *et al.* 2004, 2006, Schuel & Burkman 2005).

Inside the oviduct, the concentration of AEA is higher in the isthmus than in the ampulla. This is due to a critical balance between AEA synthesis and its degradation along the oviduct that creates locally an appropriate 'anandamide tone' that has been correlated with normal embryo development, oviductal transport, implantation, and pregnancy (Wang *et al.* 2006). However, until now, the presence of an AEA gradient inside the oviduct has not been correlated with the role played by the oviductal epithelium in sperm transport and selection. Spermatozoa become fully competent for fertilization only after a series of physiological changes referred to as 'capacitation' that occurs during their journey in the female reproductive tract (Yanagimachi 1994). Since only a few spermatozoa are able to reach the oviduct ampullary region, i.e. the physiological site for fertilization, it is obvious that capacitation *in vivo* is accompanied by a marked sperm selection to ensure a successful fertilization and embryo development. The occurrence of the endocannabinoid system (ECS) within the female reproductive tract and its suggested role in the depression of capacitation may change the classical view about sperm capacitation. Indeed, sperm capacitation has long been considered as a continuous process that once triggered leads to hyperactivated motility, acquisition of zona pellucida (ZP) binding, and fertilization competence (Yanagimachi 1994). Recent data suggest that different mechanisms promoting or suppressing capacitation may coexist, and act at different times and in different regions during the reproductive cycle (reviewed in Hunter & Rodriguez-Martinez (2004)). In mammals, during their journey within the oviduct, spermatozoa transiently adhere to epithelial cells of the isthmus tract creating a reservoir that assures the availability of competent spermatozoa at the time of ovulation (Wilmut & Hunter 1984, Hunter 1988, Smith & Yanagimachi 1990). Adhesion prolongs the sperm fertile life until poorly defined periovulatory signals induce sperm release. Several studies showed that sperm adhesion to the oviductal epithelium prevents capacitation and depresses the motility (reviewed in Hunter (2008)). However, at the time of release, adhering spermatozoa undergo rapid metabolic changes such as increase in intracellular Ca^{2+} , protein tyrosine phosphorylation, and flagellar beat frequency, which allow their migration toward the ampullary region (Gualtieri *et al.* 2005). The sperm ascension through the oviduct

may involve intermittent phases of adhesions and releases (Smith & Yanagimachi 1990, Demott & Suarez 1992, Nakanishi *et al.* 2004).

In the present paper, we report the presence of endocannabinoid receptors CB1 and CB2 on the surface of bovine spermatozoa, their localization on spermatozoa adhering to, and released from the oviductal epithelium *in vitro*, the effects of AEA on sperm viability, progressive motility, kinetics, ZP binding, and the expression of NAPEPLD in bovine oviductal epithelial cells. Overall, data support the hypothesis that endocannabinoids via CB1 play a key role in the oviductal microenvironment modulating the physiology of spermatozoa during their journey inside the female reproductive tract.

Results

Immunolocalization of CB1 and CB2 in bovine spermatozoa

Experiments were designed to localize CB1 and CB2 in bovine spermatozoa by immunofluorescence. Immunolocalization of cannabinoid receptors was carried out in the initial sperm suspension, spermatozoa bound to oviductal monolayers, and spermatozoa released from the oviduct through heparin or penicillamine ($n=4$). In the initial sperm suspension, CB1 was localized as a bright spot at the level of the sperm neck (Fig. 1a). Differently from CB1, CB2 was localized in the rostral region of the head, in the middle piece, and slightly on the tail (Fig. 1b). In control samples pretreated with corresponding specific blocking peptides, the fluorescent signal was undetectable (Fig. 1c and d). In spermatozoa bound to oviductal monolayers, CB1 was localized on the sperm neck (Fig. 2a), as in the initial population, whereas CB2 was preferentially localized in a dotted pattern restricted to the rostral region of the sperm head (Fig. 2b) and more lightly on sperm middle pieces. The different distributions of CB2 in the rostral domain of spermatozoa bound to the oviductal epithelium could be due to a redistribution of the receptor in response to sperm-oviduct adhesion. Oviductal cells were not labeled in both cases (Fig. 2). Bovine spermatozoa can be released *in vitro* from oviductal monolayers by heparin and penicillamine, which have been previously validated as powerful inducers of sperm release (Talevi & Gualtieri 2001, Talevi *et al.* 2007). Spermatozoa released by penicillamine had the same CB1 immunolocalization observed in the initial sperm population (compare Figs 1a and 3a). Interestingly, spermatozoa released by heparin were unlabeled (Fig. 3b). In spermatozoa released by penicillamine (Fig. 3c) or heparin (Fig. 3d), CB2 was localized as in the initial population.

Figure 1 Immunolocalization and western blots of CB1 and CB2 in bovine spermatozoa. Localization of CB1 (a) and CB2 (b) on initial sperm suspension. Corresponding specific blocking peptides (c and d). Bar, 10 µm. Anti-CB1 recognized a single band of ~72 kDa M.W. (e, lane 1) while the anti-CB2 recognized a single band of ~57 kDa (f, lane 1). Controls with the corresponding specific blocking peptides (e, f, lane 2).

Western blot analysis

In the extracts of the initial population, anti-CB1 recognized a single band of ~72 kDa M.W. (Fig. 1e, lane 1), while the anti-CB2 recognized a single band of ~57 kDa (Fig. 1f, lane 1; $n=4$). Controls with the corresponding specific blocking peptides were negative (Fig. 1e, f, lane 2), demonstrating the specificity of the immunoreactivity in both samples. Western blot analysis of spermatozoa released with the disulfide-reductant penicillamine or the sulfated glycosaminoglycan heparin ($n=4$) showed that in penicillamine-released spermatozoa, the CB1 antibody recognized a single band of ~72 kDa (Fig. 3e, lane 3), whereas in heparin-released spermatozoa the CB1 signal is less represented (Fig. 3e, lane 1), confirming previously results obtained with immunolocalization analysis. Parallel silver-stained

gel demonstrated that the same amount of proteins was present (Fig. 3f). Controls with the corresponding specific blocking peptides were negative (Fig. 3e, lanes 2 and 4).

Effect of AEA on sperm viability and progressive motility

To understand whether AEA affects sperm viability and progressive motility, spermatozoa were treated with 2, 5, and 10 µM AEA ($n=4$). Sperm viability was not affected, whereas 5 and 10 µM AEA caused a rapid and persistent decrease in sperm progressive motility (Fig. 4). Sperm motility decreased from 60 to 28% after 5 min of AEA exposure at 5 µM (Fig. 4a), whereas at 10 µM the motility decreased from 55 to 15% (Fig. 4b). These data show that after AEA treatment, all spermatozoa are viable but most of them lose the capacity to move progressively.

Figure 2 Immunolocalization of CB1 (a) and CB2 (b) in bovine spermatozoa bound to oviductal monolayers. Bar, 10 µm. Insets, half light-half fluorescence micrographs of CB localization. Bar, 20 µm.

Figure 3 Immunolocalization of CB1 (a, b) and CB2 (c, d) in spermatozoa released by penicillamine (a, c) and heparin (b, d). Bar, 10 μ m. Western blot of CB1 (e) in spermatozoa released by heparin (lanes 1 and 2) and penicillamine (lanes 3 and 4). Controls with the corresponding specific blocking peptides (lanes 2 and 4).

Effect of AEA on sperm kinetics

To determine the AEA effects on sperm motility, spermatozoa were exposed to 2, 5, and 10 μ M AEA ($n=4$). AEA at 5 (Fig. 5) and 10 μ M (Fig. 6) induced a rapid and persistent decrease in kinetic parameters. However, the reduction in sperm kinetics was significant only for curvilinear velocity (VCL; Fig. 6a) and straight-line velocity (VSL; Fig. 6b) at 10 μ M. Although at 5 μ M the depression of sperm kinetic parameters was not significant (Fig. 5), data indicate that the effects of AEA were reversible as all values were recovered compared with controls at 40 min.

AEA effect on sperm–oviduct interaction

Recently, Gervasi *et al.* (2009) showed that nanomolar concentrations of AEA inhibited sperm–oviduct binding and induced sperm release *in vitro*. In the present study, AEA was tested at 2 nM and 5 μ M ($n=4$). Quantitative analysis showed that after 30 min of treatment, AEA 2 nM had no effects on sperm–oviduct binding (Fig. 7a, control 99 ± 23 versus AEA 88 ± 17), whereas AEA 5 μ M slightly decreased the number of spermatozoa bound to explants compared with controls (Fig. 7a, control 99 ± 23 versus AEA 81 ± 16). Moreover, AEA both at 2 nM and 5 μ M was unable to induce a release of spermatozoa adhering to explants (Fig. 7b, control 194 ± 43 versus AEA 2 nM 203 ± 36 , AEA 5 μ M 198 ± 32).

AEA effect on sperm–ZP binding

One of the effect of AEA on the sperm physiology is to depress sperm capacitation. This experiment ($n=4$) has been designed to understand whether a) AEA depresses sperm–ZP binding, and b) heparin-capacitated spermatozoa, which lack a CB1 immunoreactivity, are still responsive to AEA. Results demonstrated that ZP binding was significantly depressed by AEA (control, Fig. 8b; AEA, Fig. 8c) and increased by heparin (Fig. 8). Interestingly, AEA was unable to depress ZP binding if spermatozoa were capacitated by pretreatment with heparin (Fig. 8a).

RT-PCR analysis of NAPEPLD mRNA

To investigate whether the bovine oviduct was able to synthesize endocannabinoids, total RNA was isolated from epithelial cells recovered from ampulla and isthmus and subjected to RT-PCR analysis of NAPEPLD. Data showed that both regions express mRNAs for NAPEPLD with similar intensity (Fig. 9).

Discussion

In mammals, among millions of spermatozoa ejaculated, only a few are able to enter the oviduct and transiently adhere to epithelial cells lining the isthmus. Adhesion to the oviduct prolongs the sperm fertile life, suggesting the presence of active controls for sperm selection and storage inside the female reproductive tract to

Figure 4 Progressive motility of spermatozoa treated with AEA 5 (a) and 10 μ M (b). Significance versus control: * $P < 0.05$; ** $P < 0.01$.

ensure a successful fertilization and embryo development. The presence of the ECS and the discovery of an AEA gradient within the mammalian oviduct (Wang *et al.* 2006) suggest the intriguing possibility that endocannabinoids are involved in the complex mechanisms that regulate sperm storage, capacitation, and selection within the oviduct.

In the present paper, we studied the presence of endocannabinoid receptors on bovine spermatozoa, the effects of AEA on sperm physiology, and the ability of oviductal epithelial cells to synthesize endocannabinoids. Main results demonstrated that 1) CB1 and CB2 are present on bovine spermatozoa; 2) AEA does not influence sperm viability, whereas it induces a rapid decrease in sperm motility and kinetic parameters; 3) AEA does not affect sperm interaction with oviductal cells; 4) spermatozoa released from the oviduct through heparin, a powerful capacitating agent, showed a decreased detection in CB1; 5) AEA reduces sperm-ZP binding, but 6) fails to do so if spermatozoa were capacitated with heparin; 7) oviductal epithelial cells express NAPEPLD, the main enzyme involved in AEA synthesis. Taken together, these data suggest that endocannabinoids in the oviductal microenvironment could modulate the motility and capacitation of spermatozoa during their journey inside the female reproductive tract.

Several evidences suggest that ECS is active inside the reproductive tract of mammals (Taylor *et al.* 2007), and AEA has also been identified in human reproductive fluids (Schuel *et al.* 2002a, 2002b). An ECS has been demonstrated in human (Rossato *et al.* 2005, Francavilla *et al.* 2009), boar (Maccarrone *et al.* 2005), mouse (Ricci *et al.* 2007, Sun *et al.* 2009), and bovine (Gervasi *et al.* 2009). In mice, ECS modulates sperm motility

during epididymal maturation, and high levels of endocannabinoids have been detected in mouse spermatozoa isolated from the caput of the epididymis, where they are functionally immotile, while low levels have been found in those isolated from the cauda where they acquire motility (Ricci *et al.* 2007, Cobellis *et al.* 2010). Inside the oviduct, the concentration of AEA is higher in the isthmus than in the ampulla. In the oviduct, a critical balance between AEA synthesis and its degradation creates locally an appropriate 'anandamide tone' that has been correlated with normal embryo development, oviductal transport, implantation, and pregnancy (Wang *et al.* 2006). However, until now, it is not known whether the presence of an oviductal AEA gradient plays a role in sperm transport and selection.

In the present paper, the presence of CB1 and CB2 in bovine spermatozoa has been demonstrated by both immunocytochemistry and western blot analysis, confirming a previous report by Gervasi *et al.* (2009). The different localizations of receptors detected herein could be due to the use of different antibodies in the immunolocalization procedure.

Figure 5 Kinetic parameters of spermatozoa treated with AEA 5 μ M. Significance versus control: * $P < 0.05$.

Figure 6 Kinetic parameters of spermatozoa treated with AEA 10 µM. Significance versus control: * $P < 0.05$; ** $P < 0.01$.

During the last years, experimental data have shown that endocannabinoids negatively influence sperm motility in several mammalian species (Schuel *et al.* 2002a, 2002b, Rossato *et al.* 2005, Cobellis *et al.* 2006, Aquila *et al.* 2009, Francavilla *et al.* 2009). In human spermatozoa, the effect of AEA on the depression of motility has been demonstrated at both nanomolar (Aquila *et al.* 2009) and micromolar concentrations (Rossato *et al.* 2005), even though in the latter case the drop of motility was associated with a loss of sperm viability (Rossato *et al.* 2005, Rossato 2008). In bovine spermatozoa, observational analysis demonstrated that AEA at nanomolar concentrations does not affect motility (Gervasi *et al.* 2009). Herein, micromolar concentrations of AEA did not affect sperm viability but induced a rapid and persistent decrease in percent progressive motility and kinetic parameters. Moreover, although at 5 µM the decrease in sperm VCL, VSL, and average path velocity (VAP) was not significant, a recovery of these kinetic parameters was observed after 40 min of treatment. This is likely to be due to AEA degradation at this time, and indicates that the effect of AEA on sperm kinetics may be reversed.

Herein, the concentrations of AEA that affect bovine sperm motility and kinetics *in vitro* are higher

than those reported in reproductive fluids. Nanomolar concentrations of AEA have been reported in human seminal plasma, mid-cycle oviductal fluid (Schuel *et al.* 2002a, 2002b), as well as in plasma of pregnant women where an AEA level < 2 nM has been shown to be required at the beginning of implantation to assist in uterine receptivity (El-Talatini *et al.* 2009). However, we do not know the levels of AEA inside the bovine oviduct and, moreover, in other species, the levels reported for plasma and whole oviduct fluids are average concentrations. Spermatozoa adhering to the oviductal cells are likely to be exposed to microenvironments containing concentrations of oviductal secretions higher than those reported in whole bovine ampullary and isthmic fluids (Parrish *et al.* 1989, Lapointe & Bilodeau 2003, Bergqvist & Rodríguez-Martínez 2006). Moreover, gametes are able to directly influence their own microenvironment. In fact, in the porcine species, spermatozoa and oocytes selectively modulate the oviductal secretory proteome (Georgiou *et al.* 2005). Here, we have shown that bovine epithelial cells express mRNAs for NAPEPLD, the main enzyme involved in AEA synthesis. Spermatozoa adhering to the oviductal cells could be exposed directly to the site of AEA production, and the local concentration of AEA might be higher than that found in the whole oviductal fluid. In mouse oviduct, a longitudinal gradient of AEA is created by a high concentration of NAPEPLD in the isthmus and a high concentration of FAAH, the main enzyme for AEA degradation inside the ampulla (Wang *et al.* 2006). In this respect, we hypothesize that a transversal AEA gradient is present between the site of AEA production, i.e. the epithelial cell where spermatozoa are attached, and the central region of the oviduct lumen. The presence of a higher AEA concentration in the isthmus (Wang *et al.* 2006), i.e. in the sperm-reservoir, raises the hypothesis that endocannabinoids have a role during the sperm ascension along the oviduct. In this respect, AEA has been reported to inhibit *in vitro* sperm-oviduct binding, and promotes sperm release (Gervasi *et al.* 2009). However, in the

Figure 7 Effects of AEA on sperm-oviduct binding (a) and release (b).

Figure 8 Effects of AEA, heparin (HEP), and heparin plus AEA (HEP+AEA) on sperm-ZP binding (a). Data are shown as percent of sperm-ZP binding respect to control that is assumed as 100%. Sperm-ZP binding is depressed by AEA and enhanced by heparin. AEA does not depress sperm-ZP binding when spermatozoa are capacitated by heparin. (b) *In vitro*-matured oocyte inseminated with Hoechst-labeled control spermatozoa. (c) *In vitro*-matured oocyte inseminated with Hoechst-labeled spermatozoa pretreated with AEA. Bar, 20 μm. Significance versus control: * $P < 0.05$; ** $P < 0.01$.

present study, we observed that AEA has no effects on sperm-oviduct binding and sperm release, even at a concentration of 5 μM. These data are in agreement with several evidences showing that release of spermatozoa bound to the oviductal reservoir is promoted by capacitation (Lefebvre & Suarez 1996, Gualtieri *et al.* 2005, Talevi *et al.* 2007, Talevi & Gualtieri 2010), whereas AEA has been shown to depress this process, at least in human and boar (Rossato *et al.* 2005, Maccarrone *et al.* 2005). Herein, sperm-ZP binding experiments demonstrated that AEA depresses sperm capacitation also in the bovine species.

Heparin and penicillamine are powerful inducers of the release of bovine spermatozoa adhering to *in vitro*-cultured oviductal epithelium, and have been suggested to represent physiological signals for release of spermatozoa sequestered in the oviduct (Talevi & Gualtieri 2001, Talevi *et al.* 2007). Both release inducers act on spermatozoa and trigger capacitation-related changes (Gualtieri *et al.* 2005, Talevi *et al.* 2007). Although both inducers trigger capacitation, we showed that only spermatozoa capacitated by penicillamine are able to revert this process (Gualtieri *et al.* 2009). Interestingly, CB1 was not detectable in heparin-released spermatozoa by both immunocytochemistry and western blot analysis, whereas, in spermatozoa released by

penicillamine, CB1 had the same immunolocalization observed in the initial sperm population. The lack of CB1 immunoreactivity in heparin-released spermatozoa could be due to a remodeling of the sperm surface induced by capacitation. Interestingly, sperm-ZP binding experiments showed that heparin-capacitated spermatozoa failed to respond to AEA, thus indicating that the observed lack of immunoreactivity was associated with a loss of functionality of the CB1 receptor.

In conclusion, data here reported support the hypothesis that ECS has an active role during the sperm transport within the female reproductive tract. Secretion of AEA from epithelial cells might contribute to the oviduct sperm-reservoir function, prolonging the sperm fertile life through the depression of motility and capacitation. Capacitation signals, such as heparin, that promote sperm release might remodel the sperm surface and cause a loss of the sperm sensitivity to AEA.

Materials and Methods

Chemicals

BSA (fraction V), penicillamine, heparin (sodium salt, purified from porcine intestinal mucosa; H3393), protease inhibitor cocktail (P2714), AEA, estradiol (E_2), FSH, LH, and Medium 199 (M4530) were purchased from Sigma Chemical Company; FCS, gentamycin, amphotericin B, HEPES, and sodium bicarbonate were purchased from Gibco. Anti-CB1 and anti-CB2 rabbit antibodies and the corresponding specific blocking peptides were purchased from Cayman Chemical (Inalco, Milan, Italy). Reagents and buffers for SDS-PAGE, including the molecular weight Precision Plus Protein Standards, were purchased from Bio-Rad. ImmunoPure Standard ABC Staining kit and BCA Protein Assay kit were purchased from Pierce (Milan, Italy).

Figure 9 RT-PCR analysis of *NAPEPLD* mRNA expression in bovine oviductal epithelial cells. Ist, isthmus; Amp, ampulla.

Nitrocellulose (0.45- μ m pore size; PROTRAN) was purchased from Whatman (Dassel, Germany). Reagents and water for preparation of salines and culture media were all cell culture tested.

Preparation of oviductal epithelial cells

Oviducts were collected at the time of slaughter and transported to the laboratory in Dulbecco's PBS (DPBS) supplemented with 50 μ g/ml gentamycin at 4 °C. Laminae of epithelial cells were recovered from oviducts of single animals by squeezing and cultured at 39 °C, 5% CO₂ in air, 95% humidity, in M199 supplemented with 50 μ g/ml gentamycin, 1 μ g/ml amphotericin B, and 10% FCS. Bovine oviductal epithelial cells were either used as swimming-everted vesicles, referred to as explants, within 24 h of culture, or as confluent monolayers. To this end, explants were initially cultured in 10-cm Petri dishes (Falcon; Becton Dickinson, Milan, Italy) for 24–48 h, and then transferred into four-well tissue culture plates (Nunc, Roskilde, Denmark) with 12 mm, gelatin-coated, German glass round cover slips on the well bottom or on gelatine-coated 10-cm Petri dishes. Fresh media changes were performed every 48 h. Cell confluence was attained in about 7–10 days, and monolayers were used within 24 h after attainment of cell confluence. Within each experiment, oviductal monolayers from a single individual were washed three times in TALP (Parrish *et al.* 1989), modified as described in Paula-Lopes *et al.* (1998), and left in this medium until sperm addition (0.5–1 h).

Sperm preparation

Frozen bovine semen (0.5-ml straws; $\sim 40 \times 10^6$ spermatozoa per straw; motility after thawing R70%), obtained from Semen Italy (San Giuliano Saliceta, Modena, Italy), was used in all experiments. Straws were thawed in a water bath at 38 °C for 30 s, and 0.5 ml of semen was either labeled with 10 μ g/ml Hoechst 33342 for 5 min at 39 °C, 5% CO₂ in air, or directly washed in 10 ml and then in 5 ml TALP with or without BSA (BSA-free TALP), by centrifugation at 170 *g* for 10 and 5 min respectively. After resuspension in fresh medium, recovered spermatozoa were assessed for concentration and percent motility using a hemocytometer placed on a microscope stage heated to 39 °C.

Sperm–oviduct adhesion and release

Sperm suspensions recovered after centrifugation in TALP were added to bovine oviductal monolayers cultured on gelatine-coated, 12 mm, round cover slips in NUNC four-well plates in 750- μ l TALP at a concentration of $1\text{--}1.5 \times 10^6/\text{cm}^2$, and incubated at 39 °C, 5% CO₂ in air, 95% humidity, for 30 min. At the end of incubation, monolayers were extensively washed with TALP to remove unbound spermatozoa. A series of monolayers with adhering spermatozoa were air-dried and processed for immunocytochemical analysis, whereas parallel monolayers were treated for sperm release by incubation with freshly prepared 0.1 mM penicillamine or with 100 μ g/ml heparin for 30 min. At the end of treatment, released spermatozoa were collected and fixed for immunocytochemical and western blot analysis.

Effect of AEA on sperm–oviduct interaction

To detect the effect of AEA on sperm–oviduct binding, sperm suspensions were added to explants in four-well plates at a concentration of $1\text{--}2 \times 10^6$ motile spermatozoa/well in 750 μ l TALP added or less with AEA 2 nM, 5 μ M, and incubated at 39 °C, 5% CO₂ in air, 95% humidity, for 30 min. At the end of incubation, unbound spermatozoa were removed by extensive washings with TALP, and cocultures were fixed to quantify the number of adhering spermatozoa as previously described (Gualtieri *et al.* 2009). To detect the effect of AEA on sperm–oviduct release, explants were inseminated in TALP as above, and after unbound sperm removal, cocultures were treated with AEA 2 nM, 5 μ M, or TALP alone, and analyzed.

Localization of CB1 and CB2 receptors in bovine spermatozoa by immunocytochemistry

Initial and released sperm suspensions were diluted to $5 \times 10^6/\text{ml}$ in DPBS, smeared on glass slides, allowed to air dry, fixed in cold methanol (–20 °C) for 5 min, washed three times for 10 min in PBS, and then processed for immunocytochemistry. To this aim, slides were treated 5 min at room temperature in 1% BSA, 0.1% Tween 20, in PBS, incubated 1 h at 37 °C in anti-CB1 or anti-CB2 rabbit antibodies (1:50; Cayman Chemical) in PBS, washed three times for 10 min in PBS, incubated in the darkness for 2 h at 37 °C in FITC-conjugated goat anti-rabbit IgG (1:600), washed again three times for 10 min in PBS, mounted, and observed at a Nikon TE 2000 fluorescence microscope. Images were acquired using a Nikon DS-cooled camera head DS-5Mc connected to a Nikon DS camera control unit DS-L1 using the same exposure conditions. Specificity controls were performed through omission of the primary antibody or through preincubation of anti-CB1 or anti-CB2 rabbit antibodies with the corresponding specific blocking peptide (Cayman Chemical; antibody/peptide 1/5) and subsequent incubation of slides in this mixture.

SDS-PAGE and western blot analysis of CB1 and CB2 receptors

Spermatozoa were centrifuged twice at 180 *g* for 10 min in BSA-free sperm-TALP (170×10^6 ml for each sample), resuspended in 50 mM Tris (pH 7.4) and protease inhibitor cocktail, and centrifuged at 800 *g* for 10 min. The pellet was resuspended 1:1 v/v in lysis medium containing 50 mM Tris (pH 7.4), 2 mM EDTA 0.4% Triton X-100, and protease inhibitor cocktail left on ice for 30 min and centrifuged at 13 000 *g* for 8 min at 4 °C. Protein concentration in the supernatants was determined with BCA Protein Assay kit (Pierce) and stored at –20 °C. Aliquots of supernatant containing 15 μ g of protein were subjected to SDS-PAGE (9% gels) transferred to 0.45- μ m pore nitrocellulose membranes, incubated overnight at 4 °C with anti-CB1 or anti-CB2 rabbit antibodies (1:250; Cayman Chemical) in Tris-buffered saline with 5% nonfat dry milk, and then for 1 h at room temperature in HRP-conjugated goat anti-rabbit IgG (1:2000). Specificity controls were performed through preincubation of anti-CB1 or

anti-CB2 rabbit antibodies with the corresponding specific blocking peptide (Cayman Chemical; antibody/peptide 1/5). The nitrocellulose membranes were developed using an ECL detection system (Pierce). In experiments on released spermatozoa, two parallel gels were prepared; one was electroblotted and the other was silver-stained to ascertain that the same amount of proteins was applied.

Evaluation of sperm viability, progressive motility, and kinetics

Sperm viability was evaluated at a bright field light microscope with the eosin/nigrosin test (Tartaglione & Ritta 2004). Progressive motility and kinetics, i.e. VCL, VSL, VAP, were evaluated by Sperm Class analyzer (SCA Microptic S.L., Barcelona, Spain) with the following software setting: frame rates: 25 frames/s; number of frames: 10 frames/object; velocity limit for slow spermatozoa: 25 $\mu\text{m/s}$; velocity limit for medium spermatozoa: 35 $\mu\text{m/s}$; velocity limit for rapid spermatozoa: 50 $\mu\text{m/s}$; minimal linearity 50%; straightness for progressive fast spermatozoa 70%. In all AEA experiments, spermatozoa were washed with BSA-free TALP to avoid the formation of BSA-endocannabinoid complexes, and controls were added with the same concentration of vehicle present in AEA-treated samples. Sperm suspensions in BSA-free TALP were divided into aliquots and treated with AEA 2, 5, and 10 μM , or TALP alone. Samples were loaded into a Makler chamber and analyzed on a heated stage at 37 °C at 5, 10, 20, and 40 min after treatment, at a Nikon TE 2000 inverted microscope connected to a Basler Vision Technology A312 fc camera with a negative phase contrast 10 $\times$ objective. To avoid sperm attachment to the bottom of the chamber, samples were diluted 1:1 v/v with TALP containing a double concentration of BSA at the time of analysis. For each time point, at least 200 cells and four fields were acquired and analyzed.

Oocyte collection and in vitro maturation

Ovaries were collected at the time of slaughter and transported to the laboratory in 0.9% NaCl solution supplemented with 50 $\mu\text{g/ml}$ gentamycin at 37 °C. Cumulus–oocyte complexes (COCs) were then collected by aspiration of individual follicles with an 18-gauge needle in M199, 50 $\mu\text{g/ml}$ gentamycin, 1 $\mu\text{g/ml}$ amphotericin B, and 10% FCS. COCs were matured in the same medium supplemented with 2 $\mu\text{g/ml}$ E_2 , 0.1 IU/ml FSH, and 10 IU/ml LH (Sigma) for 24 h at 39 °C, 5% CO_2 in air, 95% humidity.

Sperm–ZP binding assays

Hoechst-labeled sperm suspensions recovered after centrifugation in BSA-free TALP were pretreated as follows: 1) TALP 20 min plus AEA 5 μM 10 min; 2) 100 $\mu\text{g/ml}$ heparin 20 min plus AEA 5 μM 10 min; 3) 100 $\mu\text{g/ml}$ heparin 20 min plus vehicle 10 min; 4) TALP 20 min plus vehicle 10 min as control. *In vitro*-matured COCs were denuded by vortexing in M199 supplemented with 0.5 mg/ml polyvinyl alcohol and 50 $\mu\text{g/ml}$ gentamycin. The denuded oocytes were placed in groups of 20–25 in four-well plates in 500 μl BSA-free TALP.

Oocytes were inseminated for 30 min at a concentration of 1×10^6 motile spermatozoa/well with the sperm suspensions described above and in the presence of the same concentration of reagents used in the sperm pretreatment. At the end of the incubation, oocytes were rinsed five times in the same medium using flexipet adjustable handle set (Cook Italia SRL, Milan, Italy) with 140- μm denuding pipette to dislodge loosely bound spermatozoa, fixed for 1 h at room temperature in 2.5% glutaraldehyde in PBS, washed in PBS, and mounted on glass slides. Oocytes were compressed under the cover slips to visualize all bound spermatozoa in a single focus plane, and images were acquired using a Nikon Eclipse TE 2000 inverted microscope under fluorescence, by means of Nikon DS-cooled camera head DS-5Mc and NIS AR software (Nikon, Florence, Italy).

RT-PCR analysis of NAPEPLD mRNA

Total RNA was isolated from epithelial cells recovered from the isthmus and the ampulla of bovine oviducts using Nucleo Spin RNA II (GE Healthcare, Amersham, Bucks, UK) according to the manufacturer's recommendations.

First-strand cDNA was synthesized from 5- μg total RNA, using random primers and reverse transcriptase provided by Super Script First-Strand Synthesis System for RT-PCR (Invitrogen). After first-strand synthesis, PCR was performed with the following oligonucleotide primer couples: 5'-GGTCTT-CACCAAGAACATGG-3' (forward), 3'-TCTTTACAGCCTCTT-CTGGG-5' (reverse) for a product length of 999 nucleotides. The primers were designed in Primer-BLAST on the NAPEPLD bovine mRNA sequences available in NCBI (*Bos taurus* NAPEPLD, mRNA, complete cds gi|61555755|gb|BT021908.1| [61555755]). Amplifications were performed using Dream-Taq DNA polymerase (Fermentas, Vilnius, Lithuania). PCR was performed as follows: 95 °C for 2 min (initial denaturation) and 30 cycles at 94 °C for 45 s, 50 °C for 30 s, and 68 °C for 1.5 min. PCR products were separated on a 1.2% (w/v) agarose gel, stained with ethidium bromide, and recorded under u.v. light with a digital Camera. cDNAs were cloned into a pCR II vector (Invitrogen Life Technologies) and sequenced by PRIMM (Naples, Italy). All sequence information was processed using BlastN program.

Statistical analysis

The data are presented as mean $\pm$ s.d. Overall, analysis was performed by the estimate model of ANOVA (SAS/STAT 1988) followed by the Tukey's honest significant difference test for pairwise comparisons when overall significance was detected. Results expressed as percentages were modified by arcsine transformation to normalize the data.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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