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Abstract

The oviduct acts as a functional sperm reservoir in many mammalian species. Both binding and release of spermatozoa from the oviductal epithelium are mainly modulated by sperm capacitation. Several molecules from oviductal fluid are involved in the regulation of sperm function. Anandamide is a lipid mediator involved in reproductive physiology. Previously, we demonstrated that anandamide, through activation of the cannabinoid receptor type 1 (CB1), promotes sperm release from bovine oviductal epithelial cells, and through CB1 and the transient receptor potential vanilloid 1 (TRPV1), induces sperm capacitation. Herein we investigate co-activation between CB1 and TRPV1, and Ca^{2+} influx as part of the mechanism of action of anandamide during sperm release from oviductal cells. Our results indicate that in the absence of Ca^{2+} anandamide failed to release spermatozoa from oviductal epithelial cells. Additionally, sperm release promoted by cannabinoid and vanilloid agonists was abolished when the spermatozoa were preloaded with BAPTA-AM, a Ca^{2+} chelator. We also determined Ca^{2+} levels in spermatozoa preloaded with FURA2-AM co-cultured with oviductal cells and incubated with different cannabinoid and vanilloid agonists. The incubation with different agonists induced Ca^{2+} influx, which was abolished by CB1 or TRPV1 antagonists. Our results also suggest that a phospholypase C (PLC) might mediate the activation of CB1 and TRPV1 in sperm release from the bovine oviduct. Therefore, our findings indicate that anandamide, through CB1 and TRPV1 activation, is involved in sperm release from the oviductal reservoir. An increase of sperm Ca^{2+} levels and the PLC activation might be involved in anandamide signaling pathway.

Introduction

The mammalian oviduct acts as a functional sperm reservoir providing an environment that allows maintenance and competition of spermatozoa for fertilization of the oocyte. In many species, after mating, spermatozoa attach to the epithelial cells in the isthmus, lower region of the oviduct, by a specific cell-cell interaction (Suarez, 2008). This binding to the oviduct extends the sperm lifespan and delays sperm capacitation until ovulation-associated signals induce sperm release, allowing their transit to the ampulla, upper region of the oviduct, where fertilization takes place (Suarez, 2008). The exposure of spermatozoa to different environments, from the male to the female reproductive tract, activates a series of structural and biochemical changes that lead to fertile spermatozoa (Suarez, 2008; Chang, 1951). Altogether these changes are known as sperm capacitation. Adherence to the oviduct plays a key role in the selection of spermatozoa, and completion of capacitation is prompted shortly before ovulation, predominantly by Ca^{2+} influx into bound spermatozoa (Hunter, 2012).

The role of the oviductal epithelia in maintaining sperm function has been widely demonstrated. Dobrinski and collaborators found that equine spermatozoa bound to oviductal epithelium or membrane vesicles maintain low levels of cytoplasmic Ca^{2+}, compared to free-swimming sperm cells (Dobrinski, 1997). Recently, it has been shown that the adhesion of boar sperm to oviductal epithelial cells *in vivo* or *in vitro* is a mechanism for selection of sperm with low levels of tyrosine phosphorylation (Luno et al. 2013). Furthermore, sperm viability can be extended *in vitro* by incubating spermatozoa with isthmic epithelium (Murray et al. 1997; Gwathmey et al. 2006). Moreover, human and equine spermatozoa incubated with oviductal membrane vesicles reach a capacitated state more slowly than those incubated in capacitating medium alone (Dobrinski et al. 1997;

Murray et al. 1997). It has been proposed that sperm viability is maintained by preventing capacitation and its concomitant rise in cytoplasmic Ca^{2+}.

Several evidences indicate that lipid mediators serve as important signaling molecules during fertilization and early pregnancy. Among these lipid messengers, eicosanoids, lysophosphatidic acid, and endocannabinoids are well recognized as molecules that participate in reproductive events (Wang et al. 2005; Ye et al. 2010; Bari et al. 2011).

Altogether, the endocannabinoids, the enzymes that synthesize and degrade them, the putative membrane transporter of endocannabinoids and the receptors that are activated by endocannabinoids, are components of the endocannabinoid system. The participation of this system in reproductive physiology has been widely demonstrated (Karasu et al. 2011; Meccariello et al. 2014). *N*-arachidonoylethanolamide or anandamide (AEA) is an endogenous lipid that is agonist of the cannabinoid receptors type 1 and 2 (CB1 and CB2) and the transient receptor potential vanilloid type 1 (TRPV1). Anandamide is synthesized from membrane phospholipids, released to the cytoplasm and can reach the extracellular space through a putative transporter. Despite alternative ways, the enzyme fatty acid amide hydrolase (FAAH) is the principal enzyme that degrades AEA.

CB1 belongs to a family of G-protein-coupled receptors known to regulate responses to stimulation in somatic cells by modulating second messenger systems like Ca^{2+} and K^{+} channels, adenylate cyclase, phospholipases, and tyrosine kinases (Demuth et al. 2006; Wang et al. 2006). Interestingly, similar second messengers have been involved in sperm function such as capacitation, motility, and acrosome reaction (Darszon et al. 1999; Visconti et al. 1999).

TRPV1 is a ligand-gated, nonselective cationic channel that is activated by molecules derived from plants, such as capsaicin, and also by stimuli like heat or protons (Ross, 2003; Wang et al. 2006). It has been reported that AEA activates TRPV1 and the ligand-receptor interaction occurs through a cytosolic binding site triggering an increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and/or activation of protein kinases (Wang et al. 2006).

CB1 and TRPV1 receptors can exert either opposite or similar functions in the same physiological and pathological conditions, and can crosstalk when expressed in the same or in neighboring cells. In a model of nerve-injury, it has been reported that the constitutive activity of CB1 is required to maintain TRPV1 in a sensitized state (Fioravanti et al. 2008).

The endocannabinoid system has been characterized in both oviduct and sperm cells of mammals and is involved in the regulation of oviductal and sperm function (Wang et al. 2006; Maccarrone et al. 2005; Francavilla et al. 2009; Karasu et al. 2011). Furthermore, AEA is present within the male and female tract suggesting that spermatozoa are normally exposed to this lipid mediator within the reproductive tract (Wang et al. 2006; Schuel et al. 2002; Maccarrone et al. 2005). Recently we have shown that the bovine oviductal fluid contains nanomolar concentrations of AEA which fluctuate during the estrous cycle, with the highest levels detected during the peri-ovulatory period (Gervasi et al. 2013). We have also found that the bovine oviduct and spermatozoa express several components of the endocannabinoid system and that physiological concentrations of AEA or its stable analog, R(+)-methanandamide (Met-AEA), regulate *in vitro* sperm release from oviductal epithelial cells by activation of CB1 but not CB2 receptors (Gervasi et al. 2009). In addition, concentrations of Met-AEA equivalent to physiological concentrations of AEA promote events related to capacitation and this effect is reversed by CB1 and TRPV1 antagonists,

suggesting the involvement of CB1 and TRPV1 receptors in the AEA signaling pathway (Gervasi et al. 2009; Gervasi et al. 2011; Osycka-Salut et al. 2012).

Taking into account that CB1 and TRPV1 are activated during sperm capacitation, and that this event has been proposed as one of the causes of sperm release from the oviductal reservoir, we aimed to study (a) the potential link between the activation of TRPV1 and CB1 receptors in sperm release from the oviduct and, (b) the participation of TRPV1 and TRPV1-mediated gating of Ca^{2+} in the mechanism of sperm release induced by AEA.

Materials and Methods

Chemical

(R)-(+)-Methanandamide (Met-AEA), arachidonoylethanolamide or anandamide (AEA) and M199 medium were from Sigma Aldrich (St. Louis, MI, USA). Gentamicin and fungizone were from GIBCO (Life Technologies, NY, USA). The selective agonist of TRPV1 capsaicin, the selective TRPV1 antagonist capsazepine (Ant-TRPV1) and the selective FAAH inhibitor URB597 were purchased from Cayman Chemical Company (Migliore Laclaustra, Buenos Aires, Argentina). The selective CB1 antagonist SR141716A (Ant-CB1) was kind gift from Sanofi-Aventis Recherche (Montpellier, France). The phospholipase C (PLC) inhibitor U73122 and selective CB1 agonist arachidonoyl-chloro-ethanolamide (ACEA) were purchased from Tocris bioscience (Bristol, UK). Fetal Bovine Serum (FBS) was purchased from (Natocor, Argentina). The intracellular calcium indicator FURA2-AM was obtained from Molecular Probes (Life Technologies, NY, USA). The cell-permeant chelator BAPTA-AM and HEPES were obtained from Sigma Aldrich (St. Louis, MI, USA). Glass wool columns for sperm selection were from MicroFiber Manville.

Salts used to prepare sperm-Tyrode's albumin lactate pyruvate (sp-TALP) medium were purchased from MERK. All other chemicals were of analytical grade.

Sperm preparation

Cryopreserved semen of 10 different bulls (15×10^6 spermatozoa/0.5 ml straw) was obtained from Cabaña LAS LILAS (Buenos Aires, Argentina), ALTA CIALE (Center of Artificial Insemination La Elisa, Buenos Aires, Argentina) and CIAVT (Artificial Insemination Center Venado Tuerto, Santa Fé, Argentina). Straws were thawed in a water bath (37 °C for 30 s). Spermatozoa were subjected to sperm selection using glass wool columns (Calvo et al. 1989) and washed by centrifugation at 800 g with Bovine Serum Albumin (BSA)-free sp-TALP (99 mM NaCl; 3.1 mM KCl; 0.4 mM NaH_2PO_4; 0.4 mM $MgCl_2.6H_2O$; 21.6 mM Na-Lactate; 10 mM HEPES; 2 mM $CaCl_2.H_2O$; 25 mM $NaHCO_3$; 1 mM Na-pyruvate; pH=7.4).

Pellets were resuspended in BSA-free sp-TALP and assessed for sperm concentration and motility using a hemocytometer mounted on a microscope stage heated at 38.5 °C.

Oviductal cell cultures

Bovine oviducts were obtained as donation from Rio de la Plata slaughterhouse (Buenos Aires, Argentina) and Camer slaughterhouse (Santiago de Chile, Chile). Cultures of oviductal epithelia were prepared as described previously by (Gervasi et al. 2009). Briefly, oviducts were collected at the time of slaughter, transported at 4 °C, cleaned of surrounding tissues and washed three times in sterile PBS at 4 °C. After that, the oviducts were cut, flushed with sterile PBS and squeezed by pressure with tweezers.

We have performed two oviduct experimental models such as the laminae of oviductal epithelium (explants of oviductal epithelial cells) or development of primary cultures of oviductal epithelium in monolayers of bovine oviductal epithelial cells (BOEC) with very similar results. The bovine oviductal epithelium used for the development of the explants or BOEC monolayers was obtained by squeezing the entire oviduct. As our previous results showed that the binding ability of explants or BOEC from the ampulla is similar to the isthmus (data not shown), entire oviducts were used for performing the oviductal cultures.

BOEC monolayers: Laminae of bovine oviductal epithelial cells from ampulla and isthmus were recovered from different animals and pools of epithelial cells from 6 oviducts were collected together. Different pools of BOEC were washed by centrifugation at 1500 g for 5 min and incubated in M199 medium supplemented with 10% of FBS (M199 medium + FBS), gentamicin (0.1 mg/ml) and fungizone (1 µg/ml) at 38.5 °C in a 5% CO_2 atmosphere. Incubations were performed in six-well tissue culture dishes with 12 mm round cover slips on the well bottom. After 48 h, BOEC were washed by centrifugation (1500 g for 5 min) and replaced in the tissue dishes. Medium was changed every 48 h. Bovine OEC in culture displayed a characteristic epithelial polygonal shape and rarely overlapped; confluence was achieved around 7 days after starting the culture in the round cover slip. Epithelial cell purity of the culture was confirmed by immunocytochemical analysis using anti-cytokeratin antibodies (data not shown).

Finally, the oviductal monolayers were washed three times in BSA-free sp-TALP and left in this medium for 60 min until co-culture with spermatozoa.

Co-cultures of BOEC and spermatozoa.

The co-cultures were performed as described previously by (Gervasi et al. 2009). Within each experiment, confluent BOEC monolayers from different pool of oviducts were inseminated with different sperm suspensions (0.5-1 x10^6 sperm/ml of BSA-free sp-TALP/well) for 60 min at 38.5 °C in a 5% CO_2 atmosphere. After that, unbound sperm were removed by washing three times with BSA-free sp-TALP and the different treatments were added for 15 min. When selective antagonists were used, the co-cultures were pre-incubated with those for 5 min. After that, control and treated wells were washed three times with BSA-free sp-TALP to remove released sperm, fixed in glutaraldehyde 2.5% v/v for 60 min at room temperature, extensively washed and the round cover slips containing the cocultures were mounted on a glass slide. The number of bound sperm was determined analyzing 20 fields of 0.11 mm^2/cover slip under a phase contrast microscope (Olympus) in blinded experiments. The results were expressed as the media of the average of bound spermatozoa in a 0.11 mm^2 area. A replicate (n) in these experiments was defined by the co-culture of 1 pool of BOEC inseminated with sperm from 1 bull. All the treatments (including the control) were performed for each replicate. The number of spermatozoa bound to the BOEC in the controls depends on the replicate and it ranged between 30 and 80 per 0.11 mm^2 area.

Oviductal explants

Explants of oviductal epithelium were prepared as described previously (De Pauw et al. 2002) with some modifications. Briefly, oviducts were collected at the time of slaughter, transported at 4°C, cleaned of surrounding tissues and washed three times in sterile PBS at

4°C. After that, oviducts were cut, flushed with sterile PBS and squeezed by pressure with tweezers. Laminae of bovine oviductal epihelium (explants) were recovered from different animals, selected on the basis of ciliary beating, and pooled together. The oviductal cells were washed by centrifugation at 1500 g for 5 min and incubated in M199 medium + FBS at 38.5°C in a 5% CO2 atmosphere. After 24 h, explants were washed by centrifugation (1500 g for 5 min). The supernatant was removed, replaced by BSA-free sp-TALP (sp-TALP) and the explants were gently disaggregated into smaller clumps by passing once through a 21-gauge needle; 50 µl of each oviduct explant were seeded in 24 wells dish with 450 µl of sp-TALP and left in this medium for 60 min until sperm addition.

Co-cultures of explants with spermatozoa

The explants from pooled oviducts were inseminated with the same sperm suspension (2×10^6 sperm/ml) for 60 min at 38.5°C in a 5% CO_2 atmosphere. After that, the treatments (control, AEA, capsaicin, AEA and capsazepin) were added to the co-cultures for 15 min. Control and treated cells were fixed in glutaraldehyde 2.5% v/v for 60 min at room temperature, and then extensively washed with PBS.

Finally, 10 explants from each treatment, that have a surface area from 3000 to 100 μm^2 and at least one spermatozoon attached, were subjected to inspection in a Nikon microscope (400x magnification). Surface area was calculated using Image J software (Schneider et al., 2012). Spermatozoa bound to the explants surface were counted and results were expressed as the mean of the number of bound spermatozoa to 1 mm^2/explant.

Participation of Ca^{2+} in the regulation of sperm release by AEA

Experiment 1 was performed to evaluate the sperm release by CB1 or TRPV1 agonists from BOEC in the absence of Ca^{2+}. Co-cultures (spermatozoa bound to BOEC) were incubated in sp-TALP without Ca^{2+} and immediately AEA (1 nM), capsaicin (0.1 µM) and Met-AEA (1.4 nM) were added for 15 min. After that, co-cultures were washed, mounted to a glass slide and evaluated as described above.

Experiment 2 was performed to evaluate the sperm release by CB1 or TRPV1 agonists from BOEC using a cell permeable Ca^{2+} chelator. Spermatozoa were preloaded with BAPTA-AM (5 µM) or BSA-free sp-TALP (control) for 45 min at 38.5 °C in a 5% CO_2 atmosphere, and then washed by centrifugation at 800 g for 5 min. Co-cultures were performed with preloaded spermatozoa as described above. After 60 min of co-culture, the unbound spermatozoa were removed by washing 3 times with BSA-free sp-TALP, and then the co-cultures were incubated with AEA (1 nM), Met-AEA (1.4 nM) or capsaicin (0.1 µM) for 15 min. Then, the co-cultures were washed three times with BSA-free sp-TALP, fixed, mounted and counted as described above.

Measurement of Ca^{2+} in spermatozoa bound to BOEC

Single cell Ca^{2+} measurements were performed using an imaging microscopy recording method described previously by (Barrera et al. 2004). Bovine spermatozoa were preloaded with 2.5 µM of FURA2-AM for 40 min at 37 °C in a 5% CO_2 atmosphere. After that sperm cells were centrifuged at 800 g for 10 min, resuspended in BSA-free sp-TALP and co-incubated with BOEC for 60 min to allow binding. Then the unbound spermatozoa were discarded by washing three times with BSA-free sp-TALP and the co-cultures were incubated in the same medium. The intensity of fluorescence was recorded with an

Olympus fluorescence microscope coupled to an image acquisition system (Metafluor, Universal Imaging Corporation, v6.1). Serial images were acquired at excitation wavelength of 340 and 380 nm and detected at 510 nm, at 3 sec intervals during 7 min: 2 min in BSA-free sp-TALP (basal), and 5 min after the addition of the cannabinoid or vanilloid agonists and antagonists tested. In all experiments, a final incubation with ionomycin (10 μM) was done as control. Data were analyzed with the Metamorph software (Universal Imaging Corporation, v6.1), and changes in intensity of fluorescence represent changes in the intracellular Ca^{2+} levels. The intensity of fluorescence in the head of each spermatozoon was recorded during the 7 min of experiment, and the area under the curve (AUC) of this intensity was calculated as the difference between the AUC of 5 min of treatment and the 2 min of basal. A replicate (n) in these experiments was defined by the co-culture of one pool of BOEC inseminated with sperm of one bull. Between 80 and 350 total spermatozoa per treatment were analyzed, however only the responsive 40% to Ca^{2+} increase is shown in the results. The results are expressed as the media of the AUC of the spermatozoa per treatment.

Ca^{2+} influx assessment: co-cultures of FURA2-AM pre-incubated spermatozoa with BOEC were performed as described above. After 60 min co-incubation the co-cultures were washed three times with BSA-free sp-TALP to discard unbound spermatozoa and were incubated in BSA-free sp-TALP medium containing $LaCl_3$ (1 mM). The intensity of fluorescence in presence of AEA (1 nM) or capsaicin (0.1 μM) was recorded as described above.

Effect of CB1 and TRPV1 activation in sperm–oviduct interaction

Co-cultures were performed for 60 min and after washing, different treatments were applied for 15 min at 38.5 °C in a 5% CO_2 atmosphere as described above.

Co-cultures were incubated with URB597 (FAAH inhibitor; 5 nM) or Met-AEA (stable analogue of AEA; 1.4 nM) to evaluate the effect of an increase of endogenous AEA or the addition of exogenous AEA, respectively, on sperm release from oviductal cells. The incubation with capsazepine (0.1 μM) or SR141716A (0.1 nM) was also evaluated to study the specificity of the effect on TRPV1 or CB1 activation by AEA.

Co-cultures were also incubated with capsaicin (0.1 μM) and/or capsazepine (0.1 μM), or with ACEA (50 nM) and/or SR141716A (0.1 nM) to further evaluate the effect of TRPV1 or CB1 activation on sperm release from BOEC.

Concentration-response curves of the different cannabinoids and vanilloid agonists and antagonists were previously performed (data not shown).

To evaluate the effect of CB1 and TRPV1 participation in the sperm release by AEA, different experimental approaches were designed: a) co-cultures were pre-incubated with capsazepine (0.1 μM) and then incubated with ACEA (50 nM); b) co-cultures were pre-incubated with Ant-CB1 (0.1 nM) and then incubated with capsaicin (0.1 μM); c) To evaluate the participation of Phosphoinositol-Phospholypase C (PI-PLC) in the CB1/TRPV1 activation on sperm release from oviductal monolayers, co-cultures were pre-incubated with U73122 (10 μM) and after that incubated with capsaicin (0.1 μM) and/or ACEA (50 nM). All pre-incubations were performed during 5 min and the further incubations were performed for 15 min at 38.5 °C in a 5% CO_2 atmosphere.

Since drugs were dissolved in ethanol or dimethyl sulfoxide, control incubations were performed with the same final vehicle concentrations (0.0001% v/v).

Statistical analysis

Statistical analyses were performed using the software *Infostat 2011* (Di Rienzo J.A., Casanoves F., Balzarini M.G., Gonzalez L., Tablada M., Robledo C.W. InfoStat versión 2011. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina).

Data were expressed as mean ± SEM. Homogeneity of variances and normality was checked in all data. In order to accomplish the assumptions of parametric tests, data of Ca^{2+} content experiments were transformed using the logarithm base 2 transformation.

For the experiments of co-cultures without Ca^{2+} or pre-incubated with BAPTA-AM, comparison between groups was performed with a two-way analysis of variance (ANOVA) in blocks. Each pool of BOEC with spermatozoa was considered a block and all treatments were applied to it.

For the sperm-oviduct interaction experiments, comparison between groups was performed with a one-way ANOVA in blocks. Each pool of BOEC with spermatozoa was considered a block and all treatments were applied to it.

For the measurement of Ca^{2+} experiments, comparison between groups was performed with a nest ANOVA, the spermatozoa were considered nest to each plate of co-culture.

When the ANOVA tests were significant ($p<0.05$), multiple comparisons were performed by Tukey's honestly significant difference test. When $p<0.05$ the differences were considered to be significant.

Results

Participation of CB1 and TRPV1 on AEA-induced sperm release from oviductal epithelial cells *in vitro*.

We have previously shown that the incubation with exogenous AEA or Met-AEA, at nanomolar concentrations, induces sperm release from oviductal epithelia in bovines by CB1 activation (Gervasi et al. 2009) and that CB1 and TRPV1 from spermatozoa are activated by AEA during sperm capacitation [Gervasi et al. 2011]. As sperm capacitation has been proposed as cause of sperm release from the oviductal reservoir, we investigated the TRPV1 and CB1 activation during sperm release. Co-cultures of spermatozoa with BOEC were performed and the effect of a non-hydrolysable AEA analogue (Met-AEA) on sperm release was evaluated in the presence CB1 and TRPV1 antagonists. Figure 1A indicates that the pre-incubation with the selective CB1 antagonist SR141716A (Ant-CB1) or with the selective TRPV1 antagonist, capsazepine (Ant-TRPV1) completely inhibited the releasing effect of Met-AEA.

We also explored whether an increase of endogenous AEA levels might induce sperm release from BOEC trough activation of CB1 and TRPV1 receptors. As AEA is a lipid that can be synthesized by the spermatozoa or the epithelial cells (Francavilla et al. 2009; Gervasi et al. 2013), we incubated the co-cultures with URB597, a selective inhibitor of the enzyme FAAH that mainly degrades AEA (Piomelli et al. 2006) in order to increase the endogenous levels of AEA. Results indicate that the increase of AEA significantly reduced the number of spermatozoa bound to oviductal epithelial cells when compared to control (Figure 1B). Moreover, the pre-incubation with TRPV1 or CB1 antagonists fully reversed the effect of URB597 (Figure 1B).

The activation of TRPV1 by AEA or capsaicin, a TRPV1 selective agonist, in bovine sperm release was studied using oviductal explants as a different experimental approach. In agreement with our results obtained with monolayers, the incubation of co-cultures of sperm-oviduct explants with AEA or capsaicin for 15 min also induced sperm release from epithelial cells and this effect was reversed by a TRPV1 antagonist (Supplementary Figure).

Evaluation of TRPV1 and CB1 activation during sperm release by AEA

The simultaneous activation of TRPV1 and CB1 in the sperm release process was investigated.

Co-cultures of BOEC and spermatozoa were incubated with TRPV1 or CB1 selective agonists (capsaicin and ACEA, respectively) and antagonists. Figure 2A indicates that the incubation with capsaicin for 15 min decreased the number of spermatozoa bound to BOEC as compared to control. The pre-incubation with Ant-TRPV1 completely reversed the effect of capsaicin and the number of bound spermatozoa to the oviductal cells was similar to control (Figure 2A). Ant-TRPV1 alone did not have effect on sperm release (data not shown).

Similarly, the incubation with ACEA for 15 min decreased the number of spermatozoa bound to BOEC as compared to control (Figure 2B). As indicative of specificity, the pre-incubation of co-cultures with Ant-CB1 completely reversed the effect of the selective CB1 agonist, ACEA (Figure 2B). Ant-CB1 alone did not have effect on sperm release (data not shown).

As mentioned above, TRPV1 and CB1 receptors may crosstalk when they are expressed in the same cells. Considering that our results indicated that the incubation with separate antagonists (CB1 and TRPV1) completely reversed the effect of Met-AEA, we investigated whether the simultaneous activation of CB1 and TRPV1 is necessary to regulate sperm release by AEA. Interestingly, the effect of capsaicin was completely reversed by the pre-incubation with Ant-CB1 (Figure 2C). In addition, the effect of CB1 agonist on sperm release was completely reversed by the presence of Ant-TRPV1 (Figure 2D).

It is known that CB1 can modulate TRPV1 sensitivity to activation through intracellular signaling mechanisms that involve PLC activation leading to changes in the levels of phosphatidylinositol-4,5-bis-phosphate (PIP2), which tonically inhibits TRPV1 (Chuang et al. 2001). Thus experiments of sperm release in the presence of the PLC selective inhibitor U73122 were performed. The incubation with U73122 inhibited the effect of capsaicin suggesting that PLC activation is required to allow TRPV1 activation in this process (Figure 3A). Also the PLC inhibitor reversed the sperm release due to CB1 activation by ACEA (Figure 3B). In addition, the incubation of co-cultures with capsaicin and ACEA simultaneously diminished the number of spermatozoa bound to BOEC, and this effect was inhibited in the presence of U73122 (Figure 3C).

Evaluation of calcium as modulator of the sperm release elicited by AEA

Our results indicate that AEA promotes sperm release from oviductal cells by the activation of CB1 and TRPV1 receptors. We have previously shown that AEA also induces events related to sperm capacitation in bovines (Gervasi et al. 2011). An increase in sperm $[Ca^{2+}]_i$ occurs during sperm capacitation (Ruknudin et al. 1990) and during sperm release from the

epithelial cells (Petrunkina et al. 2001). Taking into account our results and that TRPV1 is a Ca^{2+} channel, we investigated whether Ca^{2+} ions are involved in the mechanism of action of AEA during the sperm release from oviductal cells.

This series of experiments was designed to study whether Ca^{2+} is required for inducing sperm release from BOEC by the activation of CB1 and TRPV1 receptors. Co-cultures of spermatozoa with BOEC were performed during 60 min, and then sperm release was induced by incubating with AEA, Met-AEA or capsaicin for 15 min in a media lacking Ca^{2+} ions. The addition of these cannabinoid and/or vanilloid agonists failed in inducing sperm release from the epithelial cells in the absence of external Ca^{2+} (Figure 4A). To further investigate the requirement of this ion for sperm release, co-cultures were performed with spermatozoa preloaded with the intracellular Ca^{2+} chelator BAPTA-AM. Spermatozoa preloaded with BAPTA-AM or pre-incubated only with sp-TALP (control) were co-cultured with BOEC during 60 min. After that, the co-cultures were treated with AEA, Met-AEA or capsaicin for 15 min to promote sperm release. Results, are shown in Figures 4B and C, and indicate that sperm release promoted by cannabinoid and vanilloid agonists was abolished when the spermatozoa were preloaded with BAPTA-AM. No effect on sperm motility was observed (data not shown).

Assessment of calcium content in spermatozoa in co-cultures

Our results indicated that the endocannabinoid agonists require Ca^{2+} to induce sperm release from the oviductal cells. Taking this into account, the next step was to evaluate possible changes in Ca^{2+} content of spermatozoa co-cultured with BOEC. Spermatozoa preloaded with FURA2-AM were co-cultured with BOEC and then incubated with

different cannabinoid and vanilloid agonists as explained in Materials and Methods. Data analysis indicates that, in all treatments, a subpopulation of approximately 40% of bound spermatozoa increased, at different extent, $[Ca^{2+}]_i$ (data not shown). This subpopulation of spermatozoa that increased $[Ca^{2+}]_i$ was selected and further analyzed as follow.

The addition of AEA to the co-cultures produced an increase in the sperm $[Ca^{2+}]_i$ expressed as the fluorescence ratio (340/380) when compared to control incubation (Figure 5A). Figure 5C shows a representative microphotography of the increase in $[Ca^{2+}]_i$ of spermatozoa bound to epithelial cells when treated with AEA. Furthermore, data were also expressed as the average of the area under the curve (AUC) of the 5 min treatment minus the 2 min of basal levels for each spermatozoon (Figure 5D). We also evaluated the effect of an increase of the co-culture endogenous levels of AEA on sperm Ca^{2+} levels. The incubation of the co-cultures with URB597 also induced an increase in the $[Ca^{2+}]_i$ of spermatozoa (Figure 5B and D). These results suggest that an increase of levels of AEA in the milieu produces a rise in sperm $[Ca^{2+}]_i$ in a subpopulation of spermatozoa.

Based on our results that show that the activation of CB1 and TRPV1 receptors is required to promote sperm release with AEA, we evaluated the participation of these receptors in the increase of sperm $[Ca^{2+}]_i$ elicited by this endocannabinoid. As expected, the incubation with the selective agonist of TRPV1 capsaicin also induced an increase in $[Ca^{2+}]_i$ of spermatozoa (Figure 6A and B). In addition, CB1 or TRPV1 antagonists completely inhibited the $[Ca^{2+}]_i$ elevation observed with AEA (Figure 7A, B and C). The incubation with the antagonists alone did not show any effect.

During the peri-ovulatory period, a Ca^{2+} influx occurs from the oviductal fluid to the spermatozoa bound to the oviduct. This increase takes place during sperm capacitation and causes structural and superficial changes in the sperm plasma membrane (Petrunkina et al. 2001) and may promote sperm detachment from the epithelia (Coy et al. 2012). We further investigated whether the increase in sperm $[Ca^{2+}]_i$ was by the input from the extracellular space. We performed $[Ca^{2+}]_i$ measurements in co-cultures incubated with AEA or capsaicin in the presence of the inorganic Ca^{2+} antagonist lanthanum chloride ($LaCl_3$) (Yueh et al. 1993). $LaCl_3$ blocks the Ca^{2+} influx from the extracellular space as it competes with extracellular Ca^{2+}, however it is not able to penetrate the intact cell membrane to block intracellular stores. Interestingly, $LaCl_3$ inhibited the $[Ca^{2+}]_i$ increase induced by both agonists (Table 1). The incubation with $LaCl_3$ alone did not have effect (data not shown).

Discussion

The mammalian oviduct acts as a reservoir for spermatozoa and provides an environment in which they may compete to fertilize the oocyte. Once in the oviduct, spermatozoa undergo capacitation that is essential for a successful fertilization. Sperm-oviduct interaction is important for *in vivo* sperm capacitation and is a tightly regulated process influenced by the local microenvironment. *In vivo*, a functional sperm reservoir is mainly located in the distal region of the isthmus and the utero-tubal junction. To study the sperm-oviduct interaction in cattle, several experimental models are used including the laminae of oviductal epithelium (explants of oviductal epithelial cells) or development of primary cultures of oviductal epithelium in monolayers of oviductal epithelial cells cultured in adequate media (De Pauw et al 2002; Gualtieri et al. 2000). In our laboratory we have

developed two models of *in vitro* sperm-oviductal cells co-cultures in order to study the participation of molecules from the oviductal fluid, such as AEA, in the regulation of the sperm fertilizing ability (Gervasi et al. 2013; Osycka-Salut et al. 2012). In this work we mainly used primary cultures of oviductal epithelial cells, and the results in sperm release were reinforced by using a second experimental approach, laminae of oviductal epithelium.

Several molecules from oviductal and follicular fluids are involved in the regulation of *in vivo* sperm capacitation (Hunter, 2012; Coy et al. 2012). Anandamide from oviductal fluid fluctuates during the estrous cycle, and the highest values are detected during the peri-ovulatory period (Gervasi et al. 2013). Nanomolar levels of AEA were also detected in follicular fluids (Gervasi et al. 2013), suggesting that during ovulation the mature follicle may contribute to oviductal AEA levels and creates an endocannabinoid gradient conducive to the regulation of sperm function within the oviduct.

Anandamide is a lipid mediator that acts through CB1 activation in the regulation of sperm release from the oviduct in bovines (Gervasi et al. 2009). In this study we present evidences that indicate that AEA also acts through TRPV1 receptors in this process. Moreover, our results indicated that the activation of CB1 and TRPV1 receptors and the participation of a PLC are involved in the sperm release from oviductal epithelia by AEA. Our results also suggest that AEA, through CB1 and TRPV1 activation, induces a sperm intracellular Ca^{2+} increase in a subpopulation of spermatozoa.

The increase of Ca^{2+} in spermatozoa is an event that is closely associated with sperm capacitation (Visconti et al. 2009; Ruknudin et al. 1990). This process promotes the removal of sperm proteins and molecules such as binder of sperm proteins that may induce the release from the oviductal reservoir (Hung and Suarez, 2012). It has been shown that

the TRPV1 agonist capsaicin promotes sperm Ca^{2+} increase in boar spermatozoa (Bernabo et al. 2010), and bicarbonate induces CB1 and TRPV1 receptor migration into lipid microdomains in capacitating conditions (Botto et al. 2010) suggesting also an important role of these receptors in the regulation of the sperm function. We have previously shown that AEA induces events related to sperm capacitation through the activation of TRPV1 and CB1 receptors in bovines (Gervasi et al.2011). In addition, our previous results also indicated a possible cooperation between both receptors, since the activation of CB1 and TRPV1 by AEA is involved in nitric oxide production during bull sperm capacitation (Osycka Salut et al. 2012).

In the present work we show that the activation of both, CB1 and TRPV1 receptors, is required for AEA to induce sperm release. This is supported by our results that show that the effect on sperm release observed with a selective agonist of one receptor was completely inhibited with a selective antagonist of the other receptor. The effect on sperm release observed when the co-cultures were incubated with capsaicin or ACEA independently can be explained by basal endogenous levels of AEA present in the media during co-culture. We have measured AEA production in either bovine spermatozoa or BOEC, and both cellular types are able to synthesize AEA *in vitro* (data not shown) in basal conditions, suggesting that a basal tone of AEA is continuously produced by oviductal cells and spermatozoa.

The activation of TRPV1 receptors is highly regulated and its activity is tonically inhibited by membrane PIP2 (Chuang et al. 2001). It is known that the enzyme PLC hydrolyses PIP2, and its activity has been related to sperm capacitation (Breitbart, 2001). In this regard, it is interesting to note that CB1 receptors are coupled to the activation of PLC

enzyme (Ho et al. 1999) and that TRPV1 activation may be modulated by the activation of CB1, especially in cells which co-express both receptors (Hermann et al. 2003). The results presented here suggest that CB1 activation is coupled to TRPV1 and that the mechanism of action of CB1 and TRPV1 is dependent on the activation of a PLC in our experimental model. Our results indicate that the incubation with a PLC inhibitor prevents the effect of the specific TRPV1 and CB1 agonists on sperm release from oviductal epithelial cells. Moreover, the fact that the binding site for AEA is extracellular in CB1 and intracellular in TRPV1 supports the idea that a sequential activation of CB1 and TRPV1 may be involved in our system. Further studies are necessary to rule out this hypothesis. Our model is consistent with other experimental models in which the cooperation between CB1 and TRPV1 receptors has been shown (Hermann et al. 2001; Evans et al. 2007).

Previous studies have indicated that phosphorylation of the TRPV1 and/or removal of PIP2 results in enhanced capsaicin-induced Ca^{2+} influx *via* the TRPV1 channel (Bhave et al. 2003; De Petrochellis et al. 2001). A complementary explanation of CB1 and TRPV1 cooperation could be that the inhibition of CB1 receptors leads to decreased TRPV1 sensitivity to agonists through alteration of the phosphorylation state of the channel. Further studies regarding this issue have to be done.

The influx of Ca^{2+} ions from the oviduct into spermatozoa that occurs in the peri-ovulatory period is one of the possible causes of the sperm release from the oviductal reservoir (Coy et al. 2012). However, specific ion channels that are activated in the sperm during the peri-ovulatory period still have to be identified. Our results show that capsaicin induces sperm release from the epithelial cells, suggesting that the non-selective cation channel TRPV1 is activated during sperm release from the oviduct. In general, the activation of these

receptors involves an increase in $[Ca^{2+}]_i$ (Ross, 2003). It is well known that the signaling pathways of Ca^{2+} are important for proper sperm function. In the present work we evaluated the involvement of Ca^{2+} in the mechanism of action of AEA during sperm release from oviductal epithelial cells. Interestingly, we have found that in the absence of Ca^{2+} AEA failed to release spermatozoa from oviductal epithelial cells. The involvement of Ca^{2+} in AEA pathway was also supported by the experiments with BAPTA-AM that showed that sperm intracellular Ca^{2+} is required for AEA in order to exert its effect. Consistent with these results, Gualtieri and coworkers (2005) demonstrated that bull spermatozoa released by heparin contain higher levels of intracellular Ca^{2+} compared to those that remain bound to the oviductal epithelium.

The determination of intracellular Ca^{2+} levels of sperm in co-culture with BOEC after the incubation with different cannabinoid agonists indicates that a subpopulation of sperm bound to BOEC increases Ca^{2+} levels. This is consistent with our previous work where we found that about 40% of spermatozoa bound to BOEC are released by treatment with physiological concentrations of AEA (Gervasi et al. 2009). In this regard, it is known that an ejaculate is a heterogeneous population of spermatozoa composed by different subpopulations and therefore sperm physiological processes do not take place simultaneously in the entire population (Florman et al. 2006). Moreover, it has been recently demonstrated that just a subpopulation of mouse spermatozoa presents correct localization of CatSper receptors and is able to undergo hyperactivated motility under capacitated conditions (Chung et al. 2014). The fact that only a subpopulation responds to incubation with cannabinoid agonists suggests that the proper functionality of the

endocannabinoid system could be part of a regulatory process that maintains sequential selection of a low number of spermatozoa with fertilizing ability.

In other biological systems the activation of CB1 is associated with the inhibition of Ca^{2+} entry (Wang et al. 2006). However our results indicated that the increase of Ca^{2+} by AEA in spermatozoa in co-culture is mediated by the activation of both TRPV1 and CB1 receptors. This is supported by the fact that the incubation with selective antagonists of these receptors prevents the augment of Ca^{2+} in the sperm elicited by AEA. Consistent with our results, the activation of CB1 receptors increases the intracellular Ca^{2+} in rat odontoblasts, and this effect is reversed by the incubation with a TRPV1 antagonist, suggesting that the entry of Ca^{2+} mediated by TRPV1 is coupled to CB1 activation (Tsumura et al. 2012).

Several studies reported the existence of at least two reservoirs of Ca^{2+} and different types of Ca^{2+}-permeable channels in the plasma membrane of spermatozoa, emphasizing the complex machinery of Ca^{2+} signaling that sperm possesses (Jimenez-Gonzalez et al. 2006). In this sense we observed that the increase of Ca^{2+} levels with AEA or capsaicin was inhibited by $LaCl_3$, an extracellular and unspecific blocker of Ca^{2+}. This supports the idea that the increase of intracellular Ca^{2+} that occurs through the activation of CB1 and TRPV1 by AEA is partly due to the entry of this ion from the extracellular space. However, we cannot discard the presence of a complementary Ca^{2+} input from intracellular stores. In this regard, previous data suggested that some highly lipophilic ligands of TRPV1 with long alkyl chains, such as capsaicin, are weak activators of TRPV1 in the endoplasmic reticulum and can only elevate $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} (Morita et al. 2006).

Collectively, the data presented in this work suggest that the stimulation of CB1 and TRPV1 receptors might be coupled to PLC activation and that AEA requires Ca^{2+} to induce

sperm release from oviductal cells (Figure 8). We propose that CB1 activation may release the tonic PIP2-mediated inhibition of TRPV1 by inducing the activation of a PLC, with a subsequent sperm Ca^{2+} influx and release from the oviductal epithelia. We have previously shown that AEA stimulates events related to sperm capacitation in bovine spermatozoa in absence of the oviductal cells [Gervasi et al. 2011]. Here we show that this endocannabinoid induces Ca^{2+} influx on the spermatozoa in co-culture with oviductal cells. However the possibility of AEA activating receptors present in the oviductal epithelial cells cannot be ruled out, and further studies are necessary to test this hypothesis.

Overall, we propose that AEA, through CB1 and TRPV1 receptors activation, is involved in sperm release from the oviductal reservoir that may be related to the acquisition of sperm fertilizing ability in the oviduct during the peri-ovulatory period. In conclusion, the present study indicates that the intracellular pathways modulated by AEA are related with the molecular events involved in activation of sperm cells thus emphasizing the role of the endocannabinoid system on sperm physiology and opening new perspectives on its therapeutic and diagnostic applications.

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Conflict of Interest

The authors have declared that no conflict of interests exist.

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Figure Legends

Figure 1. Participation of TRPV1 and CB1 receptors in the sperm release induced by AEA. Co-cultures of spermatozoa and BOEC were performed. After 1 h, co-cultures were incubated with Met-AEA or URB597 (FAAH inhibitor) and specific antagonists of vanilloid and cannabinoid receptors during 15 min. Data are expressed as media of an average number of spermatozoa in 20 fields that remained bound to an area of 0.11 mm^2 of the epithelial cells after treatments. **A.** Inhibition of the effect of Met-AEA on sperm release by the incubation with Ant-TRPV1 or Ant-CB1; n=5. **B**. Inhibition of the effect of URB597 on sperm release by the incubation with capsazepine or SR141716; n=7. Data were analyzed by block one-way ANOVA and further Tukey's post test, $a \neq b$, $p<0.05$.

Figure 2. Interaction between TRPV1 and CB1 receptors in the sperm release induced by AEA. Co-cultures of spermatozoa and BOEC were performed. After 1 h, co-cultures were incubated with different selective agonists and antagonists of vanilloid and cannabinoid receptors during 15 min. Data are expressed as media of an average number of spermatozoa in 20 fields that remained bound to an area of 0.11 mm^2 of the epithelial cells after treatments. **A.** Inhibition of the effect of capsaicin (selective agonist of TRPV1) on sperm release by the incubation with capsazepine (Ant-TRPV1), n=4. **B.** Inhibition of the effect of ACEA (selective agonist of CB1) on sperm release by the incubation with SR141716; n=5. **C.** Inhibition of the effect of capsaicin on sperm release by the incubation with Ant-CB1; n=4. **D.** Inhibition of the effect of ACEA on sperm release by the incubation with Ant-TRPV1; n=5. Data were analyzed by block one-way ANOVA and further Tukey's post test, $a \neq b$, $p<0.05$.

Figure 3. Participation of PLC in the activation of TRPV1 and CB1 receptors during sperm release from BOEC. Co-cultures of spermatozoa and BOEC were performed. After 1 h, co-cultures were incubated with different selective agonists of TRPV1 or CB1 and a PLC inhibitor U73122 during 15 min. Data are expressed as media of an average number of spermatozoa in 20 fields that remained bound to an area of 0.11 mm^2 of the epithelial cells after treatments**. A.** Inhibition of the effect of capsaicin on sperm release by the incubation with U73122; n=5. **B.** Inhibition of the effect of ACEA on the sperm release by the incubation with U73122; n=5. **C.** Inhibition of the combined effect of capsaicin and ACEA on sperm release by the incubation with U73122; n=5. Data were analyzed by block one-way ANOVA and further Tukey's post test, $a \neq b$, $p<0.05$.

Figure 4. Participation of Ca^{2+} in sperm-oviduct interaction. A. Co-cultures of spermatozoa and bovine oviductal epithelial cells (BOEC) were incubated in sp-TALP with or without Ca^{2+}. B and C) Co-cultures of spermatozoa pre-incubated with BAPTA-AM or BSA-free sp-TALP (control) and BOEC were performed. In A) and B) after 1 h of co-culture, sperm release was induced with AEA (1 nM), Met-AEA (1.4 nM) or Capsaicin (0.1 µM) during 15 min.

A) Evaluation of the effect of AEA, Met-AEA and capsaicin in Ca^{2+}-free sp-TALP. B) Evaluation of sperm release from BOEC by AEA, Met-AEA or capsaicin in spermatozoa pre-incubated with (sp-TALP) or BAPTA-AM (5 µM); C) A representative photograph of co-cultures with sperm pre-incubated with or without BAPTA-AM and after that the sperm release by AEA (scale bar: 15 µm). Data are expressed as media of an average number of spermatozoa in 20 fields that remained bound to an area of 0.11 mm^2 of the epithelial cells after treatments. Data were analyzed by two way ANOVA and further Tukey's post test. Capital letters indicate differences between treatments (control, AEA, Met-AEA and Capsaicin); lower case letters indicate differences between pre-treatments (sp-TALP and BAPTA-AM pre-incubation), n=5; $p<0.05$.

Figure 5. Single cell measurement of Ca^{2+} levels in spermatozoa during sperm-oviduct interaction: effect of AEA treatment. Spermatozoa pre-loaded with the fluorescent dye FURA2-AM were co-cultured with BOEC during 1 h. After that, co-cultures were treated with AEA, URB597 or sp-TALP (control). Images were taken during 2 min previous at the treatment (basal) and 5 min after the addition of the treatments; the intensity of

fluorescence on the sperm heads at 340 and 380 nm was recorded under a fluorescent microscope. Trace of the ratio of intensity of fluorescence of one sperm head representative of the responsive spermatozoa of each treatment (AEA or control) in **A)** and (URB597 or control) in **B).**

C. Microphotographs of co-cultures representative of changes in the intensity of fluorescence on sperm heads at basal and at 7 min of incubation with sp-TALP (Control), AEA or URB597. **D.** AUC indicates the area under the curve of the ratio of the intensity of fluorescence and was calculated as described in Material and Methods. Data were expressed as the media AUC of all the responsive spermatozoa and analyzed by nest ANOVA and further Tukey's post test, $a \neq b$, $p<0.05$. Similar results were obtained in at least five independent experiments.

Figure 6. Single cell measurement of Ca^{2+} levels spermatozoa during sperm-oviduct interaction. Effect of the specific TRPV1 activation. Spermatozoa pre-loaded with the fluorescent dye FURA2-AM were co-cultured with BOEC during 1 h. After that, co-cultures were treated with capsaicin or sp-TALP (control). Images were taken during 2 min previous at the treatment (basal) and 5 min after the addition of the treatments; the intensity of fluorescence on the sperm heads at 340 and 380 nm was recorded under a fluorescent microscope; **A.** Trace of the ratio of intensity of fluorescence of one sperm head representative of the responsive spermatozoa of each treatment (control or capsaicin). **B.** AUC indicates the area under the curve of the ratio of the intensity of fluorescence and was calculated as described in Material and Methods. Data were expressed as the media AUC

of all the responsive spermatozoa and analyzed by nest ANOVA and further Tukey's post test, a ≠ b, $p<0.05$. Similar results were obtained in at least five independent experiments.

Figure 7. Participation of TRPV1 and CB1 receptors in AEA-induced calcium increase. Spermatozoa pre-loaded with the fluorescent dye FURA2-AM were co-cultured with BOEC during 1 h. After that, co-cultures were treated with the antagonist of TRPV1 capsazepine (Ant-TRPV1), the antagonist of CB1 SR141716 (Ant-CB1), and/or AEA. Images were taken as mentioned in legends of Figure 5 and 6.

Trace of the ratio of intensity of fluorescence of one sperm head representative of the responsive spermatozoa of each treatment (AEA or AEA + Ant-CB1) in A) and, (AEA or AEA + Ant-TRPV1). **C.** Data were expressed as the media AUC of all the responsive spermatozoa and analyzed by nest ANOVA and further Tukey's post test, a ≠ b, $p<0.05$. Similar results were obtained in at least three independent experiments.

Figure 8: Proposed mechanism of action for AEA during sperm release from oviductal cells in bovines.

Supplementary Figure: Effect of anandamide (AEA) or capsaicin on sperm release from epithelium explants of bovine oviduct. Laminae of epithelia from bovine oviduct (explants) were co-cultures with spermatozoa. After 1 h, co-cultures were incubated with AEA (1.4 nM) or capsaicin (10^{-8} M) and/or pre-incubated with the selective antagonist of vanilloid receptors capsazepin (Ant-TRPV1) during 15 min. A) Effect of AEA and capsaicin on sperm release from bovine explants; reversion by pre-incubation with the

selective TRPV1 antagonist (n=6). B) Microphotography of co-culture of sperm-explants of bovine oviduct. M: 400X; Bar: 50 µm. Data are expressed as media of the number of spermatozoa bound to 1 mm2 explant. Data were analyzed by block one-way ANOVA and further Tukey's post test, $a \neq b$; $p<0.05$.

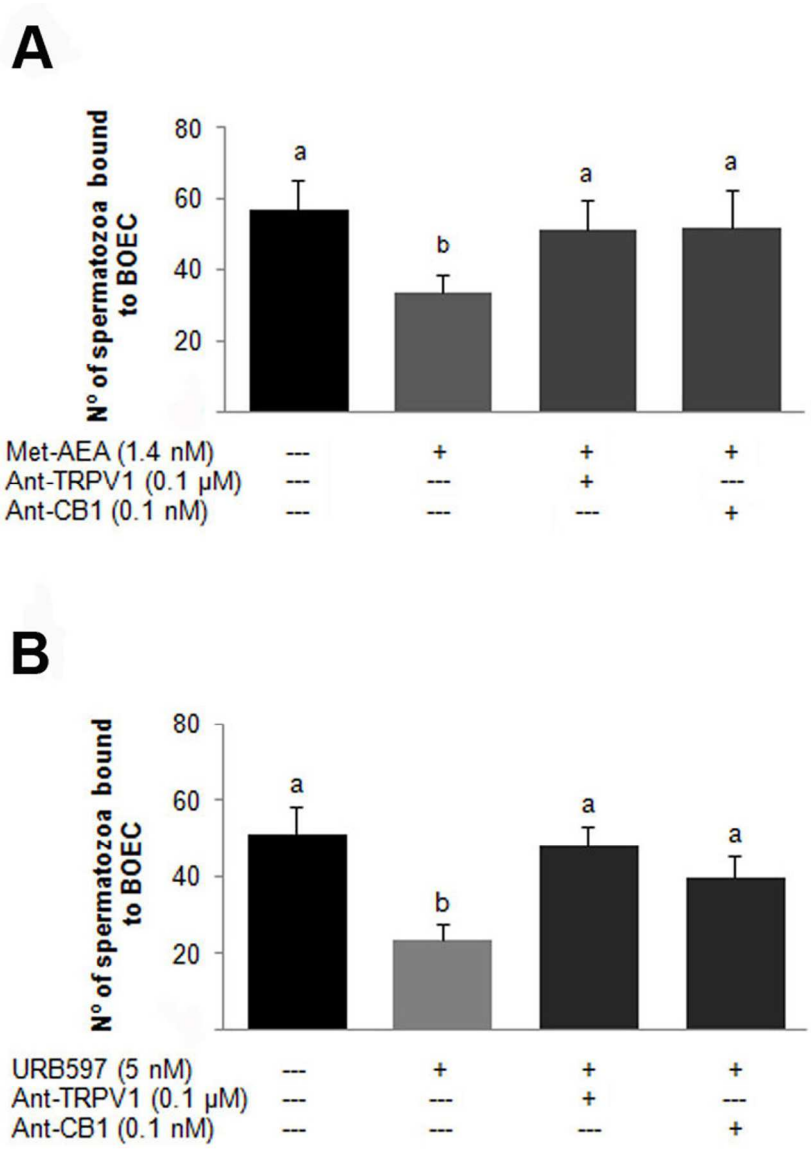

Figure 1
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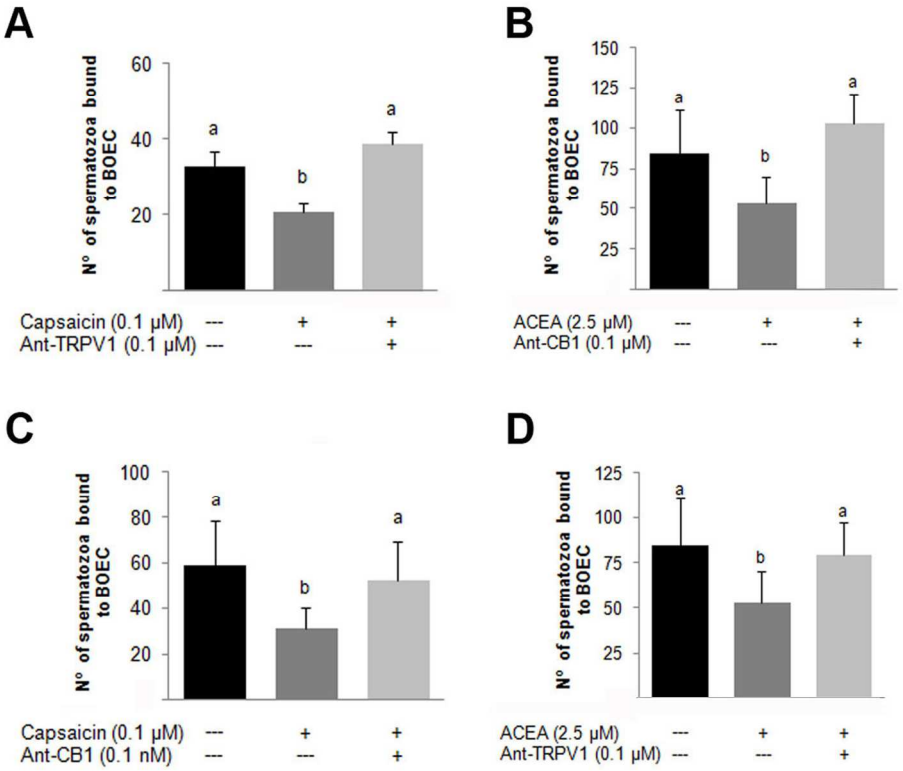

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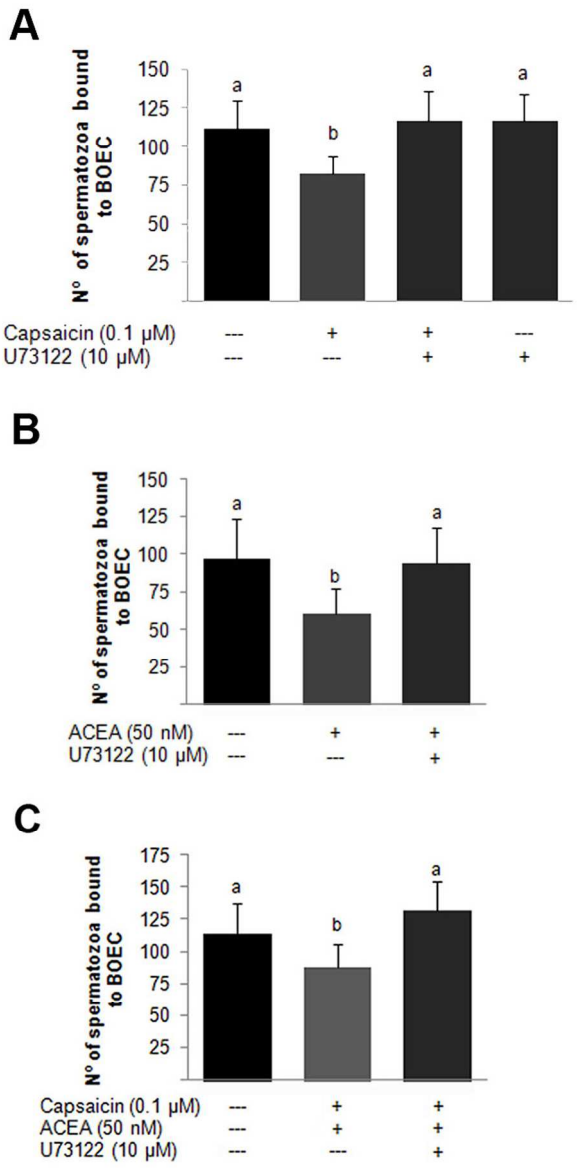

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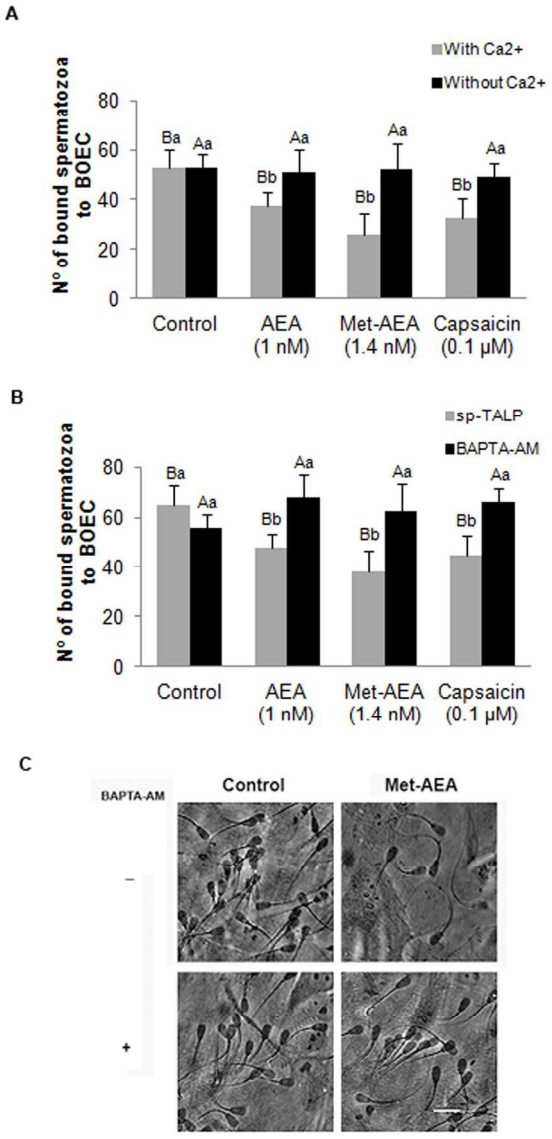

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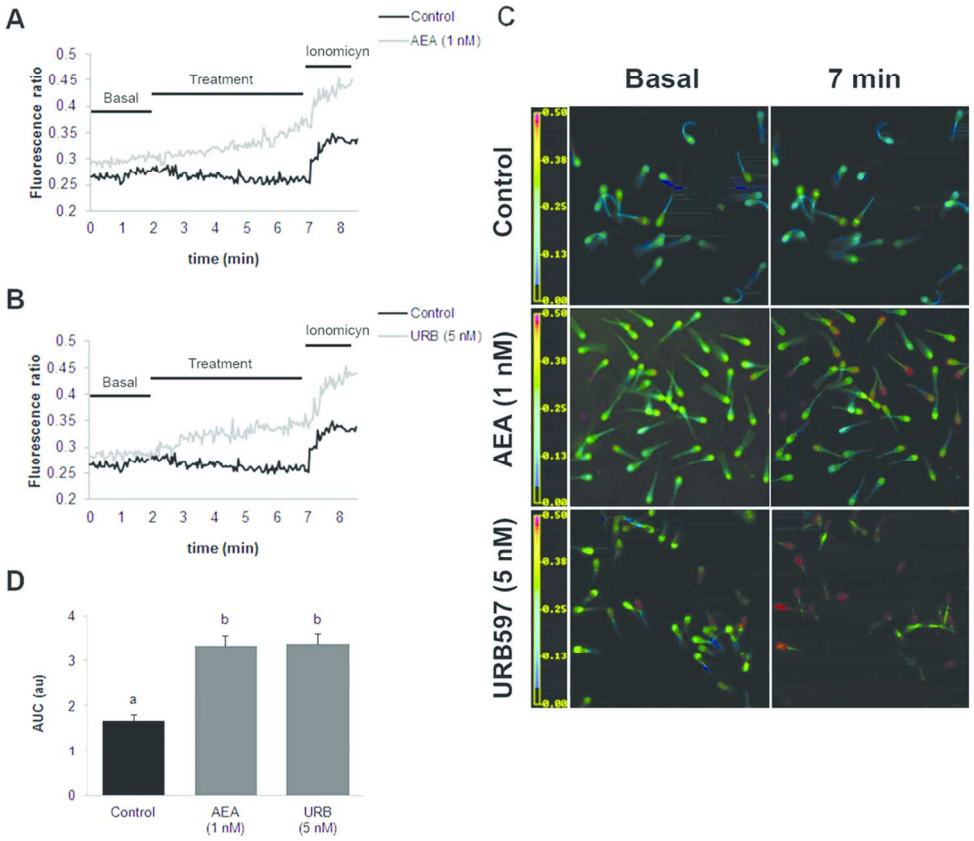

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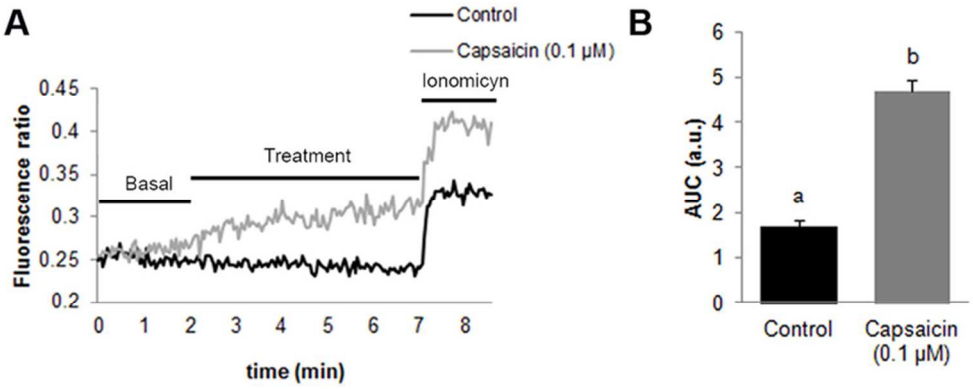

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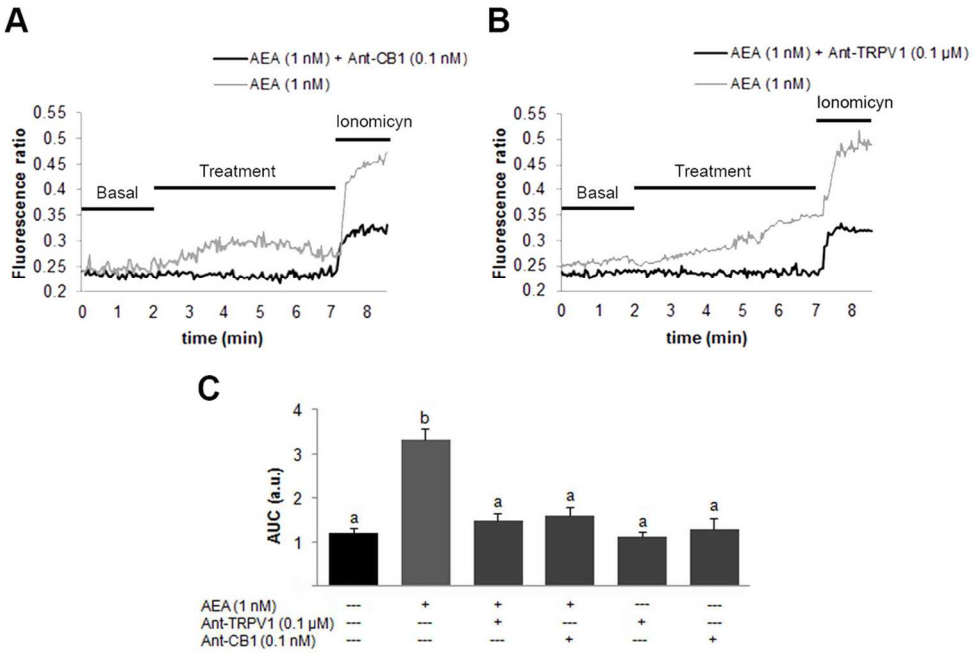

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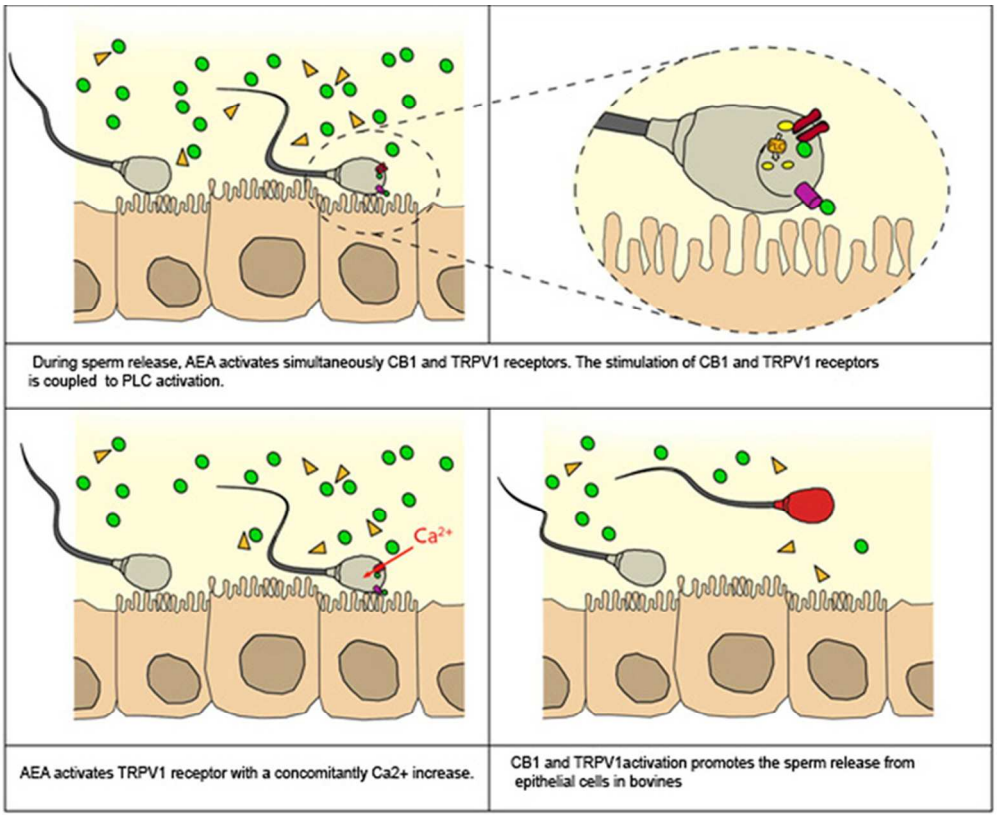

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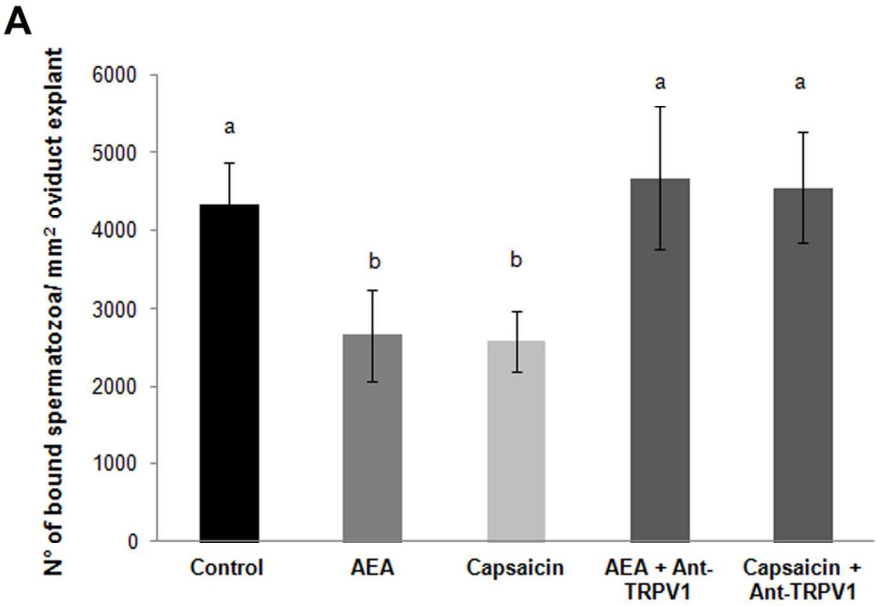

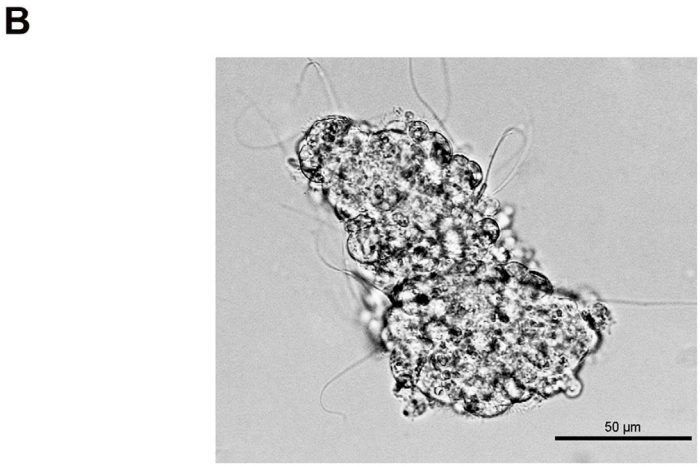

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Tables

	Intensity of Fluorescene (AUC)	
Treatment	**sp-TALP**	**sp-TALP + $LaCl_3$**
AEA (1 nM)	3.78 ± 0.83 a	1.22 ± 0.15 b
Capsaicin (0.1 µM)	5.49 ± 0.58 a	0.85 ± 0.24 b

Table 1. Effect of $LaCl_3$ in sperm $[Ca^{2+}]_i$ in co-culture with BOEC. The AUC of the intensity of fluorescence (340/380) correlates with the intracellular Ca^{2+} content. a≠b $p<0.05$ control vs $LaCl_3$.