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Fibronectin modulates the endocannabinoid system through the cAMP/PKA pathway during human sperm capacitation

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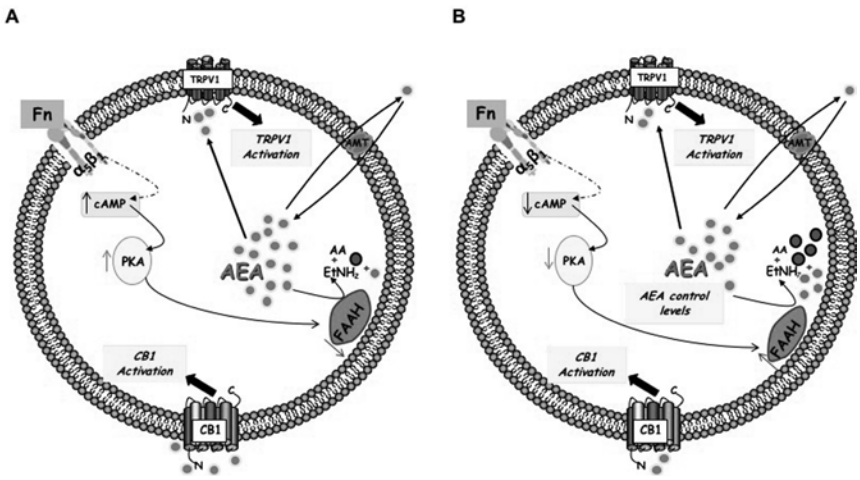
Abstract

Fibronectin (Fn) enhances human sperm capacitation via the cAMP/PKA pathway, and the endocannabinoid system participates in this process. Moreover, Fn has been linked to endocannabinoid system components in different cellular models, even though no evidence of such interactions in human sperm is available. Normal semen samples were evaluated over a four-year period. Our findings suggest that (i) the capacitating effects of Fn were reversed by pre-incubating the sperm with a cannabinoid receptor 1 (CB1) or transient receptor potential cation channel subfamily V member 1 (TRPV1) antagonist ($P<0.001$ and $P<0.05$, respectively); (ii) cooperation between CB1 and TRPV1 may exist ($P<0.01$); (iii) the activity of specific fatty acid amide hydroxylase (FAAH) decreased after 1 min ($P<0.01$) and increased after 60 min ($P<0.01$) of capacitation in the presence of Fn; (iv) the effects of Fn on FAAH activity were prevented by pre-incubating spermatozoa with a PKA inhibitor ($P<0.01$); (v) Fn modulated both the cAMP concentration and PKA activity ($P<0.05$) during early capacitation; and (vi) FAAH was a PKA substrate modulated by phosphorylation. These findings indicate that Fn stimulates human sperm capacitation via the cAMP/PKA pathway through modulation of the endocannabinoid system. Understanding the functional competence of human spermatozoa is essential for facilitating clinical advances in infertility treatment and for developing novel contraceptive strategies.

Graphical Abstract



The present study had demonstrated for the first time that Fibronectin interacts with members of the endocannabinoid system in human spermatozoa. In addition, we provide evidence that this interaction affects sperm capacitation and is mediated by the cAMP/PKA pathway.

Keywords: Fibronectin, Endocannabinoid System, cAMP/PKA Pathway, Human Sperm Capacitation.

Introduction

Capacitation comprises a series of biochemical and physiological changes that are essential to acquire fertilization capacity in mammalian spermatozoa (Chang, 1984; Yanagimachi, 1994). These changes include early, rapid events, such as the activation of cAMP-dependent pathways, intracellular pH augmentation, plasma membrane hyperpolarization (Darszon, Guerrero, Galindo, Nishigaki, & Wood, 2008), increases in intracellular Ca^{2+} levels and changes in intracellular ion concentrations (Salicioni et al., 2007), and late, slow events, such as cholesterol loss from the plasma membrane and increases in protein tyrosine phosphorylation (Signorelli, Diaz, & Morales, 2012; Visconti, 2009). Capacitation also induces functional changes in movement patterns (hyperactivation) (Yanagimachi, 1994) and ultimately confers the ability to perform the acrosomal reaction (AR) following stimulation by a physiological agonist (Hirohashi & Yanagimachi, 2018; Salicioni et al., 2007).

Capacitation takes place in the oviduct, a functional sperm reservoir that serves as an environment enabling maintenance and competence for successful oocyte fertilization. It has been suggested that some components of the oviductal fluid and molecules secreted by the oviductal and cumulus oophorus cells are involved in modulating sperm function and facilitating the acquisition of fertilization capacity (Quintero et al., 2005). One of these molecules is fibronectin (Fn), a glycoprotein composed of two similar 250-kDa subunits, that is present in follicular and oviductal fluid and the oviductal epithelium (Honda et al., 2004; Hung, Tsuiki, & Yemini, 1989; Osycka-Salut et al., 2017; Tsuiki, Preyer, & Hung, 1988). Fn binds via its RGD (Arg-Gly-Asp) domain to the cell surface through an interaction with integrins; specifically, Fn binds to integrin

$\alpha_5\beta_1$, which is present in human sperm (Fusi et al., 1996; Glander & Schaller, 1993). The binding of integrins to their ligands activates different signalling transduction pathways, leading to increased levels of intracellular Ca^{2+} or stimulation of kinase cascades such as cAMP/PKA, IP3/PKC and Src, which are involved in the regulation of sperm function (Diaz, Kong, & Morales, 2007; Lim et al., 2008; Suh & Han, 2013), particularly capacitation (Buffone, Wertheimer, Visconti, & Krapf, 2014; Lefievre, Jha, de Lamirande, Visconti, & Gagnon, 2002; Signorelli et al., 2012). These signal transduction pathways are also modulated by other molecules, such as N-arachidonoyl ethanolamide (anandamide, AEA) (Demuth and Molleman, 2006; Francavilla et al., 2009; Maccarrone et al., 2005; Osycka-Salut et al., 2012), which is also present in reproductive fluids (El-Talatini, Taylor, & Konje, 2009; Schuel, Burkman, Lippes, Crickard, Forester, et al., 2002).

AEA is an endogenous lipid agonist of the cannabinoid receptors CB1 and CB2 (Munro, Thomas, & Abu-Shaar, 1993) and vanilloid receptor type 1 (TRPV1) (Ross, 2003). Recently, a novel set of receptors that bind to AEA, GPR55 (putative CB3) (Gasperi, Dainese, Oddi, Sabatucci, & Maccarrone, 2013) and the peroxisome proliferator-activated receptors (PPARs) (Pistis & Melis, 2010) has been described. AEA is an endocannabinoid released primarily by depolarizing agents from membrane phospholipids into the interstitial space, where it performs autocrine or paracrine functions before being internalized and rapidly degraded by fatty acid amide hydrolase (FAAH), a membrane enzyme (Cravatt et al., 1996). AEA and its receptors and metabolic machinery constitute the endocannabinoid system (Maccarrone et al., 2015), which has been previously shown in human sperm (Francavilla et al., 2009).

Endocannabinoids have been postulated as reproductive biomarkers, that is, molecules with predictive significance to the reproductive potential of male and female gametes (Rapino,

Battista, Bari, & Maccarrone, 2014). Variations in the concentrations of different endocannabinoids, especially AEA, result in local changes in the female and male reproductive tracts, which in turn regulate various physiological processes, including oocyte and sperm maturation. Additionally, numerous reports have linked the endocannabinoid system to important reproductive processes, including sperm capacitation in different species (Catanzaro et al., 2011; Francavilla et al., 2009; Maccarrone et al., 2005; Osycka-Salut et al., 2012). Recent studies showed that nanomolar AEA concentrations induce sperm release from the oviductal epithelium and promote bull spermatozoa capacitation (Osycka-Salut et al., 2012). Alternatively, interactions between the endocannabinoid system and Fn have been reported. Jeske, Patwardhan, Henry, and Milam (2009) described that Fn modulates TRPV1 receptor translocation to the plasma membrane, inducing increased expression and phosphorylation at tyrosine residues in TRPV1 and in sensory neurons of the trigeminal ganglia. Moreover, Waldeck-Weiermair et al. (2008) demonstrated in endothelial cells that AEA receptors (CB1 and GPR55) interact with the transmembrane portion of integrin $\alpha_1\beta_3$, triggering different cellular responses depending on the integrin cluster formed. Notably, no data in the literature have confirmed this interaction in human spermatozoa.

We have previously demonstrated that Fn induces human sperm capacitation via the cAMP/PKA pathway. Specifically, we observed that Fn enhanced capacitation by regulating cAMP levels, PKA enzymatic activity and tyrosine phosphorylation (Martinez-Leon et al., 2015). However, it is unclear how Fn exerts these effects in human sperm. Given that both Fn and AEA have been linked to sperm capacitation and that Fn has been reported to interact with the endocannabinoid system in other cell types, this work aimed to elucidate the possible association between Fn and the endocannabinoid system in the regulation of human sperm capacitation. For this purpose, we evaluated whether 1) CB1, CB2 and TRPV1 receptor activation are involved in Fn-mediated

human sperm capacitation, 2) endocannabinoid receptor cooperation plays a role in human sperm capacitation, 3) Fn regulates FAAH activity during capacitation, and 4) Fn modulates the endocannabinoid system via the cAMP/PKA pathway during capacitation.

Materials and methods

Semen collection and analysis

The Ethics Committee on Scientific Research at the University of Antofagasta approved the research presented in this manuscript. The institutional review board approved the use of all semen samples provided by human donors aged 17-30 yr. All donors signed a consent form agreeing to the use of their sperm cells for research purposes. Freshly ejaculated sperm were obtained from healthy volunteers by masturbation after 2-3 days of sexual abstinence. The semen samples were subsequently allowed to liquefy in a slide warmer for 30-60 min at 37 °C. The same person processed all semen samples using the same equipment. Analyses of semen volume, pH, sperm concentrations and sperm motility and viability were performed as described previously (Martinez-Leon et al., 2015). The mean values for semen parameters are summarized in Supplementary Table S1. Ejaculate volumes were measured using graduated pipettes, and pH values were measured using pH paper. Sperm concentrations were assessed using a haemocytometer after appropriate dilutions. Progressive motility (PR), non-progressive motility (NP), immotility (IM) and total motility (PR+NP) were measured at 37 °C. Viability of spermatozoa was evaluated via eosin–nigrosin staining. All semen samples were normal according to the World Health Organization criteria (WHO, 2010).

Media cultures

Oviductal culture was performed using M199 medium supplemented with 50 µg/ml gentamicin, 1 µg/ml fungizone and 10 % foetal calf serum (FCS) (v/v) (Gibco, Invitrogen), and oviductal incubation and development of monolayer cultures were performed using FCS (Gualtieri & Talevi, 2000). Sperm handling and co-culture experiments were performed in modified Tyrode's medium without (non-capacitating medium, NCM) or with 2.6 % BSA (A7030) and 25 mM HCO_3^- (reconstituted capacitating medium, RCM) (Sigma Chemical Co., St. Louis, MO, USA), as we described previously (Signorelli, Diaz, Fara, Baron, & Morales, 2013). Neither sperm viability nor sperm motility was affected by the absence of HCO_3^- and BSA.

Sperm suspension preparation and *in vitro* sperm capacitation

Motile sperm were separated using a double-Percoll gradient (40/80 %) (Sigma Chemical Co., St. Louis, MO, USA), as described previously (Morales & Cross, 1989). Briefly, semen aliquots were layered on the upper layer of a Percoll gradient and centrifuged for 20 min at 300 g. The pellet was subsequently resuspended in 10 ml of NCM and centrifuged at 300 g for 10 min. Finally, the sperm cells were resuspended in the appropriate medium at the necessary concentration. Approximately 5×10^6 cells/ml were incubated in RCM or NCM in the presence or absence of 100 µg/ml Fn (Millipore Corporation, Bedford, MA, USA) for different times at 37 °C in air supplemented with 5 % CO_2. The sperm were pre-incubated in the presence of the antagonists for 15 min at 37 ºC and 5 % CO_2.

Chlortetracycline (CTC) assay

Sperm capacitation status was assessed by CTC assays, as described previously (Kong, Diaz, & Morales, 2009). A CTC solution containing 750 mM CTC in a buffer of 130 mM NaCl, 5 mM

cysteine and 20 mM Tris-HCl, pH 7.8 (Sigma Chemical Co., St. Louis, MO, USA), was prepared the day of its use and was wrapped in foil at 4 ºC until use. Then, 10 µl of CTC stock solution was rapidly added to a 10-µl aliquot of capacitated sperm suspension and treated with 10 mg/ml Hoechst 33258 for 30 sec, followed by fixation in 2 % glutaraldehyde in 1 M Tris buffer, pH 7.8, for 30 sec. Twenty microliters of this suspension was subsequently placed on a slide and allowed to dry; then, a drop of DABCO (Sigma Chemical Co., St. Louis, MO, USA) mounting medium was carefully mixed with the suspension to retard fluorescence fading. A coverslip was placed on top of the slide. Cell viability was assessed using Hoechst 33258 (Sigma Chemical Co., St. Louis, MO, USA). In each sample, 200 live cells were assessed for CTC staining patterns, and the proportion of dead cells was very low in all cases (0-5 %). The following three primary CTC fluorescence patterns were identified: the F pattern, which was characterized by uniform fluorescence over the entire head and was characteristic of non-capacitated, acrosome-intact cells; the B pattern, which was characterized by a fluorescence-free band in the post-acrosomal region and was characteristic of capacitated, acrosome-intact cells; and the AR pattern, which was characterized by dull or absent sperm head fluorescence and was characteristic of capacitated, acrosome-reacted cells (Lee et al., 1987).

Preparation of sperm extracts and immunoprecipitation for FAAH

Magnetic beads (Dynabeads®, Life technologies, Carlsbad, CA, USA) bound to recombinant G protein were used to immunoprecipitate FAAH. Sixty to eighty million sperm per millilitre, obtained using different experimental protocols, were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer containing 150 mM NaCl, 50 mM Tris, 1 % sodium dodecyl sulfate (SDS), 2 mM Na_3VO_4, 50 mM NaF, 2 mM EDTA, 1 % sodium desoxycholate, 1 % NP-40, 1 mM PMSF, 10 mg/ml leupeptin, 10 mg/ml bestatin A, and 10 mg/ml aprotinin, pH 7.4 (Sigma Chemical Co.,

St. Louis, MO, USA). The sperm suspension was then sonicated (Virsonic, Gardiner, NY) with six 20-sec. 60-W bursts in 400 µl of RIPA buffer (containing approximately 200 µg of protein), followed by centrifugation for 5 min at 14000 g to remove nuclear and flagellar material. Then, the beads were incubated with anti-FAAH 27-Y antibodies (2 mg) (Santa Cruz, CA, USA) on an orbital shaker for 10 min at room temperature. The resulting bead-antibody complexes were incubated with the protein extract on an orbital shaker overnight at 4 °C. The supernatant and the immune complex were saved separately. Both were separated electrophoretically by SDS-PAGE. Afterwards, the samples were treated according to the Western blot protocol described below using a primary antibody recognizing phospho-Ser/Thr PKA substrate (pPKAs, 1:1000) (Cell Signaling Technology, Danvers, MA).

SDS-PAGE and Western blotting

For SDS-PAGE, aliquots of immunoprecipitates containing 20 µg of protein were boiled for 5 min in sample buffer (500 mM Tris-HCl, 10 % SDS, 30 % glycerol, 1 M DTT and 0.01 % bromophenol blue, pH 6.8) and then immediately placed on ice. The samples were subsequently resolved by 10 % SDS-PAGE (10 % acrylamide/bisacrylamide for the resolving gel and 5 % acrylamide/bisacrylamide for the stacking gel) in a Mini Protein Cell. After running, the gel was equilibrated in transfer buffer for 15 min, and the proteins were electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Bedford, MA, USA) at 25 V for 30 min using a mini trans-blot cell (Bio-Rad Laboratories Inc., Hercules, CA, USA). The success of protein transfer was observed by Ponceau red staining (Sigma Chemical Co., St. Louis, MO, USA). The membranes were blocked for 60 min with 5 % non-fat dry milk and 5 % BSA in phosphate buffered saline (PBS)-Tween 20 (0.1 %, v/v), washed and probed with a mouse antibody against pPKAs. Then, the membranes were washed and incubated with an HRP-

conjugated secondary antibody (Chemicon, Temecula, CA, USA), enabling trouble-free detection of immunoblotted target protein bands, without interference from denatured IgG. This procedure facilitates the detection of co-immunoprecipitated target protein bands without masking by IgG heavy (50 kDa) and light chain (25 kDa) (Abcam, ab13166, Cambridge, United Kingdom). An electrochemiluminescence (ECL) kit was used to detect the HRP-labelled proteins according to the manufacturer's instructions (Millipore Corporation, Bedford, MA, USA). Pre-stained protein standards with molecular masses ranging from approximately 10-250 kDa were used (Bio-Rad Laboratories Inc., Hercules, CA, USA). The immunoblots were recorded as digital images (In-Vivo F Pro, Bruker, Billerica, MA, USA).

PVDF membrane stripping

Blots probed for pPKAs were stripped and reprobed with an antibody against FAAH (1:200) to confirm equal protein loading. For this procedure, approximately 30 ml of stripping buffer, consisting of 2 % (w/v) SDS, 100 mM 2-mercaptoethanol, and 62.5 mM Tris, pH 6.7, was added to the membrane and placed under constant shaking for 1 h at 60 °C. The membrane was then washed 3 times for 10 min each in Tris-buffered saline (TBS), blocked with 5 % non-fat dry milk in PBS-Tween 20 (0.1 %, v/v), and probed with the abovementioned primary antibody.

Bovine oviductal cell cultures

Bovine oviductal cell cultures were prepared as described previously (Gervasi et al., 2009; Martinez-Leon et al., 2015). Bovine oviducts were collected at the time of slaughter, transported at 4 °C, cleaned of surrounding tissues and washed twice in sterile PBS at 4 °C. Then, they were cut, flushed with sterile PBS and squeezed with tweezers. Monolayers of bovine oviduct epithelial cells (BOECs) were recovered from different animals, selected on the basis of ciliary

beating, and pooled together. The BOECs were then washed by centrifugation at 1500 g for 5 min and incubated in M199 medium at 39 °C in a 5 % CO_2 atmosphere. Incubations were performed in six-well tissue culture dishes with 12-mm round cover slips on the bottom of each well. After 48 h, the BOECs were washed by centrifugation (1500 g for 5 min) and reseeded in tissue culture dishes. The M199 medium was changed every 48 h. The oviductal monolayers from the same pool of animals were washed twice in NCM after reaching confluence and were incubated in NCM for 60 min until aliquots of motile sperm were added.

Release experiment and bound sperm quantification

A release experiment was used as a method to evaluate sperm capacitation and was performed as described previously (Gervasi et al., 2009; Martinez-Leon et al., 2015). In addition, heterologous human sperm-BOEC co-cultures were prepared as described by Ellington, Broemeling, et al. (1999), Ellington, Evenson, et al. (1999), Ellington et al. (1998).

Motile sperm selected in NCM were incubated with Hoechst 33342 (1 µg/ml) for 2 min (Sigma Chemical Co., St. Louis, MO, USA). Then, the sperm cells were washed with NCM and centrifuged at 300 g for 5 min. Afterwards, 14×10^6 sperm cells/ml were incubated with BOECs at 37 °C with 5 % CO_2 for 2.5 h, the amount of time determined to be necessary for sperm binding. The medium was then removed, and the cultures were washed 3 times with NCM to remove unattached sperm. Subsequently, the co-cultures were incubated for different times under different treatments. After co-culture, the oviductal monolayers with attached sperm were fixed in glutaraldehyde (2.5 % v/v) for 60 min at room temperature, washed, and mounted on glass slides. The numbers of bound sperm were determined by analysing 20 fields per 0.11-mm^2 cover slip by fluorescence microscopy (400x) (Nikon E200, Japan). The results were expressed as the number of sperm bound to BOECs.

Total cAMP concentration

The total cAMP concentration was determined by enzyme-linked immunosorbent assay (ELISA) using the Arbor Assay Cyclic AMP Direct EIA Kit (Ann Arbor, Michigan, United States) according to the manufacturer's instructions. Briefly, 30 x 10^6 cells/ml were incubated for different periods of time. Afterwards, the sperm were lysed in the presence of 25 mg/ml IBMX (a phosphodiesterase inhibitor) to conduct the protocol as indicated by the commercial kit.

Specific enzymatic activity of PKA

The enzymatic activity of PKA was assessed by ELISA using the Arbor Assay Protein Kinase A Activity Non-Radioactive Assay Kit according to the manufacturer's instructions. Aliquots of 20 × 10^6 spermatozoa/ml obtained using different experimental protocols were lysed to conduct the protocol as indicated by the manufacturer of the ELISA kit to assess PKA enzymatic activity.

Specific activity of FAAH

Spermatozoa were resuspended in AEA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6) and sonicated for 2 min. The homogenate was stored at 4 °C. Protein concentrations were determined using the Bradford method, and 30 µg of protein was used for each protein assay (approximately 10 × 10^6 sperm). The homogenate was incubated in AEA buffer supplemented with FAAH buffer (500 mM Tris-HCl, pH 8.5), 10^{-2} M tritiated AEA solution (H^3-AEA) (Perkin Elmer, Waltham, Massachusetts, USA) and cold 10^{-2} M AEA solution at 37 °C for 30 min. Chloroform-methanol (1:1) extraction was performed after incubation, and both enzymatic reaction products, H^3-AEA and H^3-arachidonic acid (H^3-AA), were identified. The solvent was evaporated under a hood. H^3-AA separation was performed via thin-layer chromatography (TLC Sílica gel 60, Merck, Darmstad, Germany), and AA was used as a standard (Perkin Elmer, Waltham, Massachusetts,

USA). Band locations were identified after chromatography by incubating the plate with solid iodine. The plate was then cut at the heights of selected bands, and radioactivity was measured in a liquid scintillation counter (Tricarb 2800TR, Perkin Elmer, MA, USA).

Statistical analyses

Data were analysed using one-way analysis of variance and Tukey's multiple comparison test for unequal replicates using GraphPad software (6.0e, GraphPad Software, Inc., La Jolla, CA, USA). A difference between groups of $P \leq 0.05$ was considered significant. All data are presented as the mean±SEM.

Results

Fn and Met-AEA stimulate human sperm capacitation but do not have a synergistic effect

To determine whether there was a link between Fn and the AEA signalling pathway during human sperm capacitation, spermatozoa were incubated in presence of Fn and/or Met-AEA (non-hydrolysable analogue of AEA), and capacitation status was evaluated using the CTC assay. CTC analysis is useful for evaluating intracellular calcium mobilization and for correlating calcium mobilization with the capacitation status of human spermatozoa (DasGupta, Mills, & Fraser, 1993).

We have previously described (Martinez-Leon et al., 2015) that Fn stimulates human sperm capacitation by using six different approaches (CTC, heterologous co-culture of human sperm-bovine oviductal epithelial cells (sperm-BOEC), measurement of cyclic AMP levels (cAMP), PKA activity, tyrosine protein phosphorylation (p-Tyr) and AR induction by progesterone). At this time, CTC results perfectly correlate with the results of p-Tyr and all other assays performed. Thus, we decided to evaluate capacitation through the CTC assay by measuring the percentage of spermatozoa with the B pattern. Here, we observed that

when sperm were incubated in the presence of Met.AEA (RCM+Met-AEA), there was a rapid increase in the percentage of sperm with the B pattern relative to spermatozoa incubated with Fn (RCM+Fn). The increase after 1 min of incubation (8-10 %) with respect to RCM remained constant throughout the incubation period (60, 180 and 300 min). After 1080 min of incubation, there were no significant differences with respect to RCM (Fig. 1). When the cells were incubated in the presence of both molecules (RCM+Fn+Met-AEA), the effect was similar to that observed upon incubation with Fn or Met-AEA independently (Fig. 1). These results indicate that Fn and AEA stimulate human sperm capacitation. However, when we incubated sperm in the presence of both molecules, a synergistic effect was not observed, suggesting that Fn and Met-AEA act through the same signalling pathway.

Considering that Fn not only induces sperm capacitation but also can induce AR (Diaz et al., 2007) and that the most significant changes were observed at the beginning of the capacitation process (Martinez-Leon et al., 2015), we conducted all other experiments after 1 and 60 min of capacitation. As previously described, Fn and AEA do not induce AR at the selected times (Martinez-Leon et al., 2015; Rossato, Popa, Ferigo, Clari, & Foresta, 2005).

Fn stimulates human sperm capacitation through the CB1 and TRPV1 receptors

As Fn and AEA stimulate human sperm capacitation, we wanted to evaluate the impact of selective antagonists of CB1, CB2 and TRPV1 on Fn-mediated stimulation during sperm capacitation. Human spermatozoa were pre-incubated with the following endocannabinoid receptor antagonists: AM251 (for CB1), SR144528 (for CB2) and capsazepine (CZP) (for TRPV1) (Tocris Bioscience, MO, USA). Sperm were incubated in NCM or RCM with or without Fn for 1 and 60 min. The results indicated that the effect of Fn during capacitation was completely reversed in sperm pre-incubated with the TRPV1 antagonist (Fig. 2A) or the CB1

antagonist (Fig. 2B), but not the CB2 antagonist (Fig. 2C), at both time points (1 and 60 min). These results suggest that Fn induces human sperm capacitation through the endocannabinoid system, specifically by activating the CB1 and TRPV1 receptors.

Capacitated sperm are released from oviductal epithelial cells (Lefebvre & Suarez, 1996; Osycka-Salut et al., 2012). To confirm that Fn induces sperm capacitation via the CB1 and TRPV1 receptors, we prepared heterologous BOEC and human sperm cell co-cultures, as described in the Materials and Methods section, and evaluated the participation of the CB1, CB2 and TRPV1 receptors in the release of spermatozoa from the oviductal epithelium (Fig. 3).

The results indicated that the effect of Fn on sperm release from BOECs was completely reversed when sperm were pre-incubated with a TRPV1 antagonist (Fig. 3A) or a CB1 antagonist (Fig. 3B), but not a CB2 antagonist (Fig. 3C), at both time points (1 and 60 min).

Taken together, these results indicate that Fn induces sperm release from BOECs through CB1 and TRPV1 receptor activation, supporting the CTC assay results.

CB1 and TRPV1 receptors cooperate during human sperm capacitation.

Our results suggest that Fn induces human sperm capacitation via the endocannabinoid system, and this effect is mediated through the CB1 and TRPV1 receptors. When we incubated sperm with corresponding antagonists, complete reversion of the effects of Fn was observed, although we could not determine whether one receptor or both receptors might be active during sperm capacitation. Therefore, we elected to evaluate possible modulation between these two receptors during sperm capacitation.

For this purpose, different experimental approaches were designed: a) we pre-incubated sperm with 0.1 µM CZP (selective TRPV1 antagonist) followed by 10 nM arachidonyl-2-chloroethylamide (ACEA, selective CB1 agonist), and b) we pre-incubated sperm with 0.1 µM AM251 (selective CB1 antagonist) followed by 0.1 µM capsaicin (CPS, selective TRPV1 agonist). The percentage of cells exhibiting the B pattern of capacitation was evaluated through the CTC assay.

The results presented in Fig. 4A indicate that incubating spermatozoa with CPS increased the percentage of sperm exhibiting the B pattern, similar to incubation with Fn. However, pre-incubating sperm with the CB1 antagonist (RCM+CPS+AM251) blocked the stimulatory effect induced by the TRPV1 agonist (RCM+CPS) at 1 and 60 min.

Similarly, incubating spermatozoa with ACEA increased the percentage of sperm exhibiting the B pattern, as observed with Fn. However, the TRPV1 antagonist (RCM+ACEA+CPZ) blocked the stimulatory effect induced by the CB1 agonist (RCM+ACEA; Fig. 4B) at both time points (1 and 60 min).

These results suggest that simultaneous activation of the CB1 and TRPV1 receptors may be necessary for Fn-induced human sperm capacitation.

Fn modulates FAAH activity during human sperm capacitation through the cAMP/PKA pathway

FAAH is the enzyme that degrades AEA and plays a fundamental role in regulating the endocannabinoid system by controlling intracellular AEA levels. Thus, we evaluated the effects of Fn on the specific activity of FAAH during capacitation (Fig. 5A).

The specific activity of FAAH decreased by 60 % in sperm incubated in RCM relative to sperm incubated in NCM at both incubation time points. When Fn was added to sperm incubated in RCM, FAAH enzymatic activity decreased significantly at 1 min (by approximately 50 %) relative to RCM alone. However, after 60 min of capacitation, a significant increase in FAAH enzymatic activity (by approximately 200 %) was observed relative to incubation in RCM alone.

This result indicates that Fn decreases FAAH activity after 1 min of incubation, suggesting that sperm AEA levels have increased; however, Fn increases FAAH activity after 60 min of incubation, suggesting that sperm AEA levels have decreased.

We previously demonstrated that Fn modulates cAMP levels and PKA activity during human sperm capacitation (Martinez-Leon et al., 2015). Here, we determined the effects of capacitation on cAMP levels and PKA activity during the early events of sperm capacitation. Fn induced rapid increases in cAMP levels (Fig. 5B) and PKA activity (Fig. 5C) after 1 min of capacitation, whereas it significantly decreased cAMP levels (Fig. 5B) and PKA activity (Fig. 5C) after 60 min of capacitation. Notably, the modulatory effects of Fn on PKA activity correlated with its effects on cAMP levels. As our results suggested that Fn modulates cAMP levels as well as PKA and FAAH activities, we evaluated whether Fn modulated FAAH enzymatic activity through the cAMP/PKA system. We pre-incubated spermatozoa with the specific PKA inhibitor H89 (10 μM) or KT5720 (50 nM) for 30 min and assessed FAAH activity. Fig. 5A shows that both PKA inhibitors completely reversed the effect of Fn on FAAH enzymatic activity, resulting in FAAH activity levels similar to those in NCM. This effect was observed at both incubation time points.

We performed FAAH immunoprecipitation and then immunoblotting with an antibody that recognizes PKA substrates to confirm whether the cAMP/PKA pathway modulates FAAH enzymatic activity. We observed that FAAH is a PKA substrate, but its phosphorylation was

nearly undetectable after 1 and 60 min of incubation of sperm in NCM in the presence or absence of Fn (Fig. 5D). In contrast, spermatozoa incubated in RCM showed an increase in the phosphorylation level after 1 and 60 min relative to spermatozoa incubated in NCM. This suggests that when the FAAH enzyme is phosphorylated, its activity decreases (see Fig. 5A). However, FAAH phosphorylation was significantly increased in sperm incubated in RCM in the presence of Fn at 1 min relative to sperm incubated with RCM alone. This observation is consistent with the observed changes in FAAH activity (see Fig. 5A). In spermatozoa incubated for 60 min in the presence of Fn, a slight decrease in the amount of FAAH phosphorylation was observed relative to spermatozoa incubated in RCM alone (Fig. 5D), and this result was also consistent with the observed changes in FAAH enzymatic activity (see Fig. 5A).

Discussion

We demonstrated previously that Fn enhances human sperm capacitation (Martinez-Leon et al., 2015), but the mechanisms underlying the involvement of Fn in human sperm capacitation were unclear. The present study has demonstrated for the first time that Fn interacts with members of the endocannabinoid system in human spermatozoa. In addition, we provide evidence that this interaction affects sperm capacitation and is mediated by the cAMP/PKA pathway.

Previously, an interaction between the endocannabinoid system and Fn was described in both endothelial cells (Waldeck-Weiermair et al., 2008) and the central nervous system (Jeske et al., 2009). In addition, it was observed that Fn and AEA are capable of inducing sperm capacitation independently (Gervasi et al., 2016; Martinez-Leon et al., 2015). Given this background, we first tested whether there was a summative or synergistic effect between both molecules. CTC assays demonstrated that Met-AEA (a non-hydrolysable AEA analogue) induces capacitation, as does Fn. However, no synergistic effects were observed when spermatozoa were incubated with Fn

and Met-AEA (Fig. 1), suggesting that Fn and Met-AEA mediate capacitation through the same signalling pathway or through a crosstalk signalling mechanism. Given the results obtained, we evaluated whether Fn-induced sperm capacitation is mediated by the endocannabinoid system. We first assessed whether Fn-induced sperm capacitation is mediated by the AEA receptors using CTC assays and heterologous co-culture experiments. According to the results obtained using both approaches, Fn-induced sperm capacitation was mediated by the CB1 and TRPV1 receptors but not by the CB2 receptor. As observed in our study, Gervasi et al. (2016) described that pre-incubating bovine sperm with a selective CB1 or TRPV1 antagonist completely inhibited AEA release from BOECs (sperm-BOEC co-cultures), indicating that the CB1 and TRPV1 receptors participate in AEA-induced bovine sperm capacitation. A recent study described that AEA at nanomolar concentrations decreased the number of spermatozoa bound to oviduct explants and that the suppressive effect of AEA on sperm-oviduct binding was inhibited by a CB1 receptor antagonist in water buffalo (Kumar et al., 2017). However, it has been reported that the CB2 receptor does not participate in this process, which is consistent with our findings (Gervasi et al., 2011). Moreover, previous studies have demonstrated that the CB1 and TRPV1 receptors, but not the CB2 receptor, are involved in the regulation of sperm capacitation in different species (Amoako et al., 2013; Aquila et al., 2010; Francavilla et al., 2009). Agirregoitia et al. (2010) suggested that CB2 may regulate human sperm motility; however, this finding has not been confirmed by other studies.

Although it was observed that the CB1 and TRPV1 receptors mediate the effects of Fn on capacitation, we noted that antagonizing either of them completely inhibited the effects of Fn on capacitation (Fig. 2 and 3). This finding led us to question why antagonizing one receptor results in complete rather than partial inhibition of Fn-mediated capacitation and whether this inhibitory profile involves regulatory crosstalk between the two receptors, as described in previous

investigations utilizing other cell models. For example, Ahluwalia, Rang, and Nagy (2002) observed a high degree of co-localization of CB1 with TRPV1, and Ross (2003) and Hermann et al. (2003) reported that TRPV1 receptor activation in central nervous system cells depends on CB1 activation and that these two receptors have similar activation pathways. In particular, the latter group observed that CB1 agonist pretreatment significantly enhanced the effects of capsaicin at various Ca^{2+} concentrations in HEK-293 cells overexpressing both the CB1 and TRPV1 receptors but not in cells overexpressing only the TRPV1 receptor. This effect was blocked by a CB1 receptor antagonist and by inhibitors of PI3K and PLC. Additionally, this group observed that CB1 activity is tonically inhibited and that TRPV1 receptor activation is heavily regulated by PIP2 at the membrane (Chuang et al., 2001). Given the background described above and our results, we decided to evaluate the possible modulation between TRPV1 and CB1 during Fn-induced capacitation. As shown in Fig. 4, the results suggest a cooperative effect between CB1 and TRPV1. Consistent with our results, Gervasi et al. (2016) suggested that CB1 activation is coupled to TRPV1 and that the mechanisms underlying the activities of CB1 and TRPV1 are dependent on PLC activation during AEA-induced bovine sperm release from the oviductal reservoir. PLC is known to hydrolyse PIP2, and PLC activity has been linked to sperm capacitation (Breitbart, 2002). Thus, it is interesting that CB1 receptor activity is coupled to the enzymatic activation of PLC (Ho, Uezono, Takada, Takase, & Izumi, 1999), similar to integrin activation. Regarding this finding, Tvorogov, Wang, Zent, and Carpenter (2005) observed that PLC-γ1 co-immunoprecipitated with Src following Fn-induced integrin activation in fibroblasts. Based on this evidence, we believe that CB1/TRPV1 cooperation may be facilitated by Src pathway activation. Additionally, AEA binds to TRPV1 at an intracellular site and to CB1 at an extracellular site, suggesting sequential TRPV1/CB1 receptor activation may occur in human spermatozoa. Further studies are required to corroborate this hypothesis. Note that we are the first

to show that this cooperative effect exists in human sperm. More functional studies, including electrophysiological recordings, are necessary to reveal the physiological role of TRPV1 in spermatozoa and its link to the endocannabinoid system.

We previously demonstrated that Fn-mediated effects are specific and triggered by Fn-integrin $\alpha_5\beta_1$ binding (Martinez-Leon et al., 2015). In this study, the results suggest that the CB1 and TRPV1 receptors were indirectly activated through Fn-integrin $\alpha_5\beta_1$ binding during sperm capacitation. At present, it is uncertain when Fn modulates the endocannabinoid system to activate these receptors. Previous studies have reported that AEA at nanomolar concentrations stimulates bovine sperm capacitation (Gervasi et al., 2011; Osycka-Salut et al., 2012) through CB1 and TRPV1 activation and that different AEA concentrations trigger different signalling pathways and induce distinct effects, including sperm capacitation, in the female and male reproductive systems (Agirregoitia et al., 2010; Amoako et al., 2013; Bovolin et al., 2014; Schuel, Burkman, Lippes, Crickard, Mahony, et al., 2002). Therefore, the FAAH enzyme is relevant due to its essential role in regulating AEA levels (Cascio & Marini, 2015; Francavilla et al., 2009; Howlett et al., 2011; Maccarrone, 2009; Rapino et al., 2014). FAAH activity decreased significantly in capacitated spermatozoa relative to non-capacitated spermatozoa, indicating that capacitation itself regulates endocannabinoid system function. Catanzaro et al. (2011) and Gervasi et al. (2011) observed similar results in mouse and bovine models, respectively.

Data analysis also demonstrated that Fn differentially influences FAAH enzymatic activity (Fig. 5A). Specifically, Fn decreases FAAH activity during early capacitation, leading to increases in AEA levels and CB1 and TRPV1 receptor activation, simultaneously increasing the number of capacitated sperm (Fig. 1, 2 and 3). However, Fn increases FAAH activity after 60 min of

capacitation, leading to decreased AEA levels. This result is very interesting, as maintenance of AEA levels is essential for successful fertilization, as described above.

After observing that Fn induces changes in FAAH activity, we next examined how this modulation is produced. Studies utilizing other cellular models have demonstrated that FAAH activity is regulated by PKA-dependent pathways (Grimaldi, Rossi, Catanzaro, & Maccarrone, 2009; Maccarrone, 2009), and our results indicate that Fn modulates cAMP levels and PKA activity during capacitation. Thus, we sought to establish whether Fn modulates FAAH enzymatic activity during capacitation by the cAMP/PKA pathway. It must be noted that the cAMP/PKA pathway plays an essential role in capacitation and is not exclusively activated by Fn signalling. Unsurprisingly, our results indicate that FAAH activity was partially—but not completely—restored by specific PKA inhibition (Fig. 5A). We observed that Fn induces an increase in cAMP levels after 1 min of incubation coincident with a rapid increase in PKA activity and a decrease in FAAH activity. However, after 60 min, Fn decreases cAMP levels coincident with a decrease in PKA activity and an increase in FAAH activity. Rossi et al. (2007) observed similar results, determining that FSH activates numerous transcription factors and accessory proteins by phosphorylation and increases FAAH expression and activity through the cAMP/PKA pathway in Sertoli cells. Multiple studies have shown that sperm capacitation is a HCO_3^-- and Ca^{2+}-dependent process (Gadella & Harrison, 2000; Garbers, Tubb, & Hyne, 1982; Signorelli et al., 2012; Visconti et al., 1995)**.** As described previously (Diaz et al., 2007), Fn regulates intracellular Ca^{2+} levels, which could explain why this glycoprotein modulates cAMP levels. The presence of HCO_3^- and an increase in intracellular Ca^{2+} lead to the activation of soluble adenylate cyclase (SACY) as reported previously (Garbers et al., 1982; Shi & Roldan, 1995; Visconti, 2009). Notably, additional studies are needed to investigate the exact mechanisms by which Fn is able to modulate cAMP levels.

We performed FAAH immunoprecipitation to evaluate the degree of FAAH phosphorylation by immunoblotting using an antibody that recognizes PKA protein substrates. Our data completely matched the above results, showing changes in the amount of FAAH phosphorylation. Similar results have been described by Grimaldi et al. (2009), who reported that in Sertoli cells, binding of FSH to its receptor induces PKA activation, which in turn modulates FAAH activity through the phosphorylation of accessory proteins. In conclusion, these results indicate that Fn modulates FAAH activity through the cAMP/PKA pathway. In this work, we showed for the first time that FAAH activity is regulated by PKA phosphorylation during sperm capacitation. We also performed bioinformatics analysis using three different types of prediction software to determine whether FAAH contains PKA phosphorylation sites. We identified six possible PKA phosphorylation sites at serine residues using Kinasephos 2.0 software (Wong et al., 2007); nineteen possible PKA phosphorylation sites at serine residues and six phosphorylation sites at threonine residues using NetPhos 2.0 software (Blom, Gammeltoft, & Brunak, 1999); and five possible PKA phosphorylation sites at serine residues and two sites at threonine residues using PhosphoSVM software (Dou, Yao, & Zhang, 2014) (Fig. S1). This bioinformatics approach helped us understand and verify that FAAH activity is regulated by the cAMP/PKA pathway.

Considering the background and the findings presented herein, we propose that capacitation is regulated by Fn-integrin $\alpha_5\beta_1$ binding and endocannabinoid system modulation (Fig. 6). Spermatozoa contact Fn by its receptor, the $\alpha_5\beta_1$ integrin, which is present on their cell surfaces, while passing through the uterine tubes, triggering cAMP upregulation, PKA activation, FAAH phosphorylation and AEA upregulation. AEA activates the CB1 and TRPV1 receptors, promoting capacitation in human sperm. Fn subsequently decreases cAMP levels, triggering an increase in FAAH activity and a corresponding decrease in AEA levels, ensuring the proper control of sperm capacitation. Notably, control of AEA levels is extremely important because the cellular effects

of AEA depend on its concentration. In the case of sperm, AEA at nanomolar concentrations induces capacitation, whereas AEA at micromolar concentrations inhibits capacitation (Amoako et al., 2013; Aquila et al., 2010; Gervasi et al., 2016; Maccarrone et al., 2015).

In conclusion, in the present study, we provide evidence that the effect induced by Fn during human sperm capacitation is mediated by the endocannabinoid system through the cAMP/PKA pathway. These findings contribute to a greater understanding of the molecular mechanisms underlying physiological human sperm capacitation.

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Figures

Figure 1. **Effects of Fn and Met-AEA on the B pattern of human sperm during capacitation.** Human sperm were incubated in RCM in the presence (solid line/black circles) or absence of Fn (dotted line/dark grey triangles) or in the presence (dotted line/dark grey square) or absence of Met-AEA and both molecules (solid line/black diamond) for different periods of time (1, 60, 180, 300 and 1080 min). The capacitation state was evaluated using the CTC assay, as described in the Materials and Methods section. The results were obtained from twelve different donors and are expressed as the mean±SEM of the percentage of cells exhibiting the B pattern. ***P≤0.001 RCM+Fn vs RCM; ***P≤0.001 RCM+Met-AEA vs RCM; ***P≤0.001 RCM+Fn+Met-AEA vs RCM (ANOVA, Tukey's multiple comparison test).

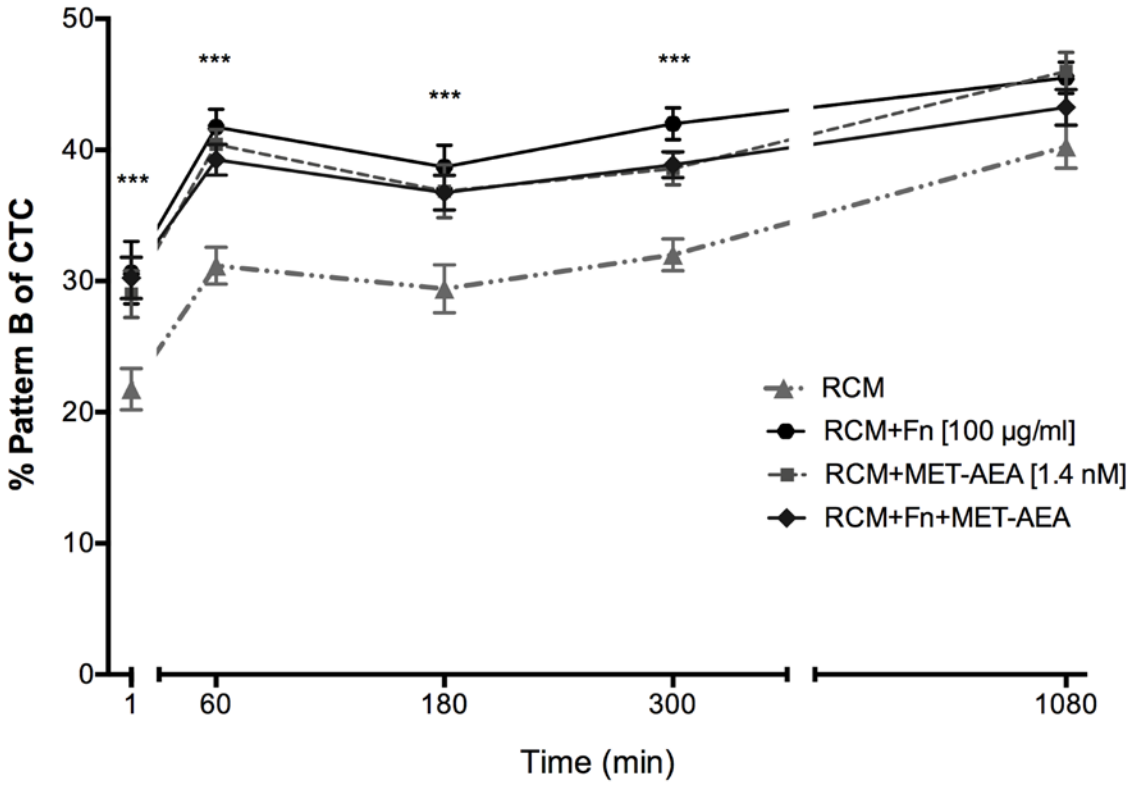


Figure 2. **Fn modulates human sperm capacitation through the CB1 and TRPV1 receptors.** Human sperm were pre-incubated in the presence or absence of the following specific endocannabinoid receptor antagonists: (A) 0.1 nM CZP (TRPV1 antagonist), (B) 0.1 µM AM251 (CB1 antagonist) or (C) 0.1 nM SR144528 (CB2 antagonist) in NCM or RCM with or without 100 µg/ml Fn, incubated for 1 min or 60 min. Capacitation status was evaluated by the CTC assay, as described in the Materials and Methods. The results were obtained from nine different donors and are expressed as the mean±SEM of the percentage of cells exhibiting the B pattern. a≠b≠c P<0.001 (ANOVA, Tukey's multiple comparison test).

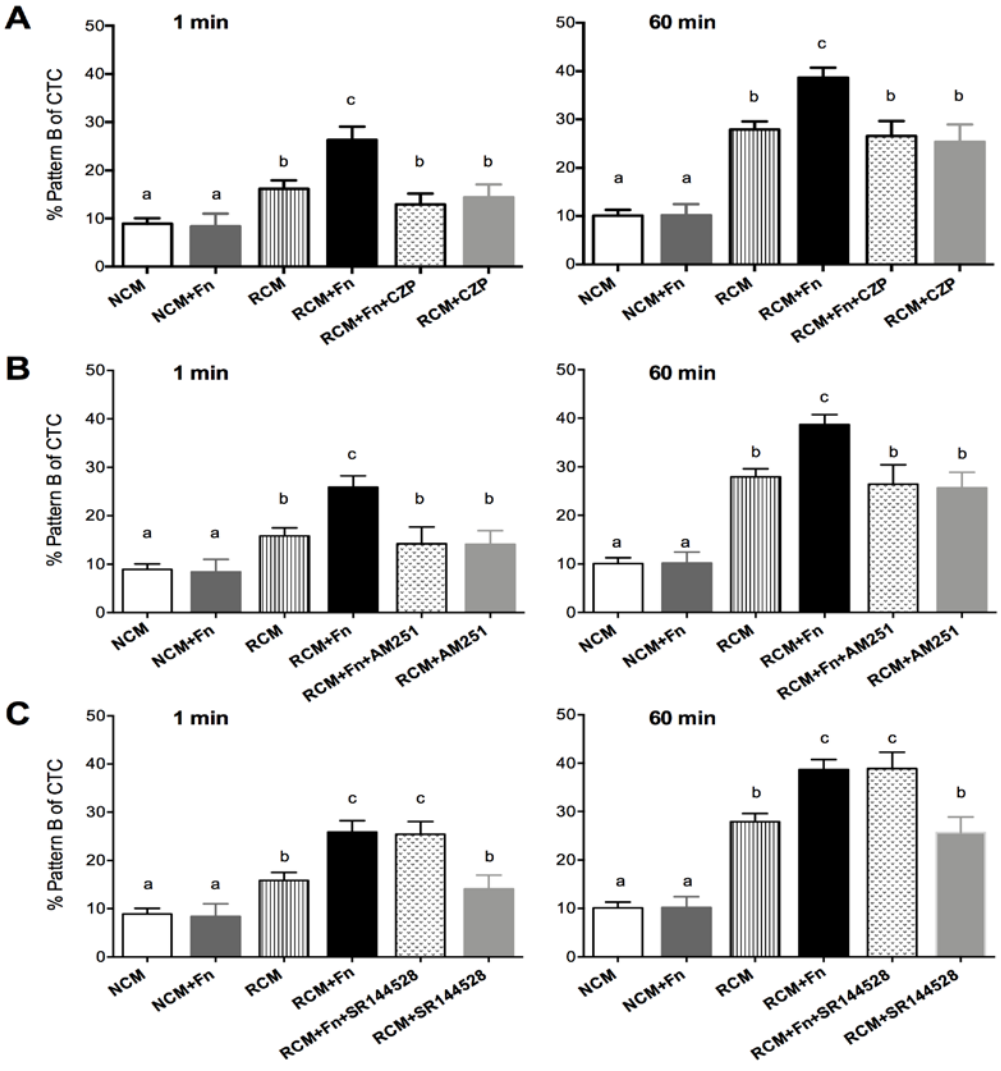


Figure 3. **Participation of CB1 and TRPV1 in Fn-induced human sperm release from BOEC monolayers.** A BOEC monolayer and 12 × 10^6 sperm/ml were co-cultured in NCM or RCM with or without 100 µg/ml Fn for 2.5 h. Human sperm were pre-incubated in the presence or absence of the following specific endocannabinoid receptor antagonists: (A) 0.1 nM CZP (TRPV1 antagonist), (B) 0.1 µM AM251 (CB1 antagonist) and (C) 0.1 nM SR144528 (CB2 antagonist), incubated for 1 min or 60 min. The bars represent the number of spermatozoa that remained attached to the BOEC monolayers, and the results are expressed as the mean±SEM of six independent experiments. a≠b≠c $P<0.05$ (ANOVA, Tukey's multiple comparison test).

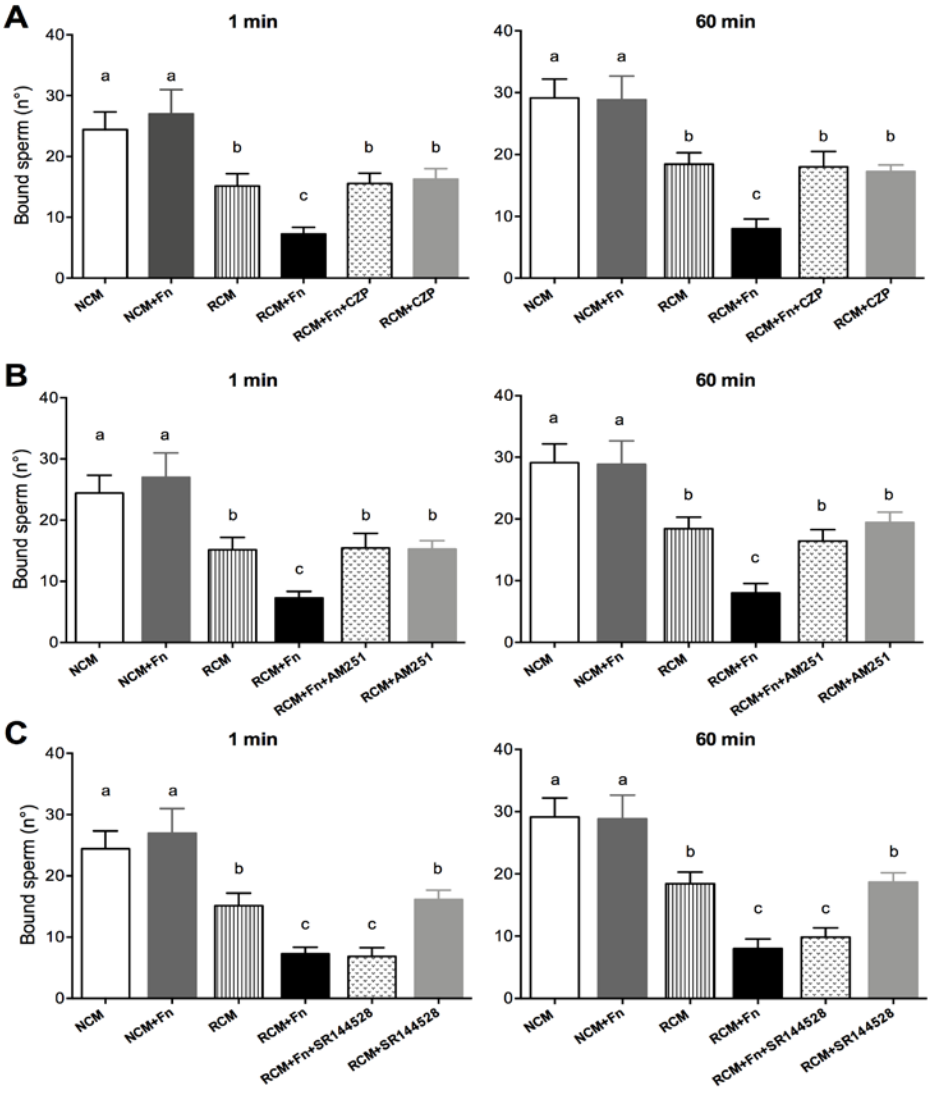


Figure 4. **The CB1 and TRPV1 receptors interact during Fn-induced human sperm capacitation**. (A) Human spermatozoa were pre-incubated in the presence of 0.1 µM AM251 (CB1 antagonist) in NCM and then incubated in the presence of 1 mM CPS (TRPV1 agonist) in RCM with or without 100 µg/ml Fn. (B) Human sperm were pre-incubated in the presence of 0.1 nM CZP (TRPV1 antagonist) in NCM and then incubated in the presence of 10 mM ACEA (CB1 agonist) in RCM with or without 100 µg/ml Fn. Human spermatozoa incubated in NCM with or without 100 µg/ml Fn were used as controls. In the CTC assay, the B pattern was identified at 1 and 60 min. The bars indicate the percentages of sperm exhibiting the B pattern in four independent experiments. Data are expressed as the mean±SEM. a≠b≠c $P<0.01$ (ANOVA, Tukey's multiple comparison test).

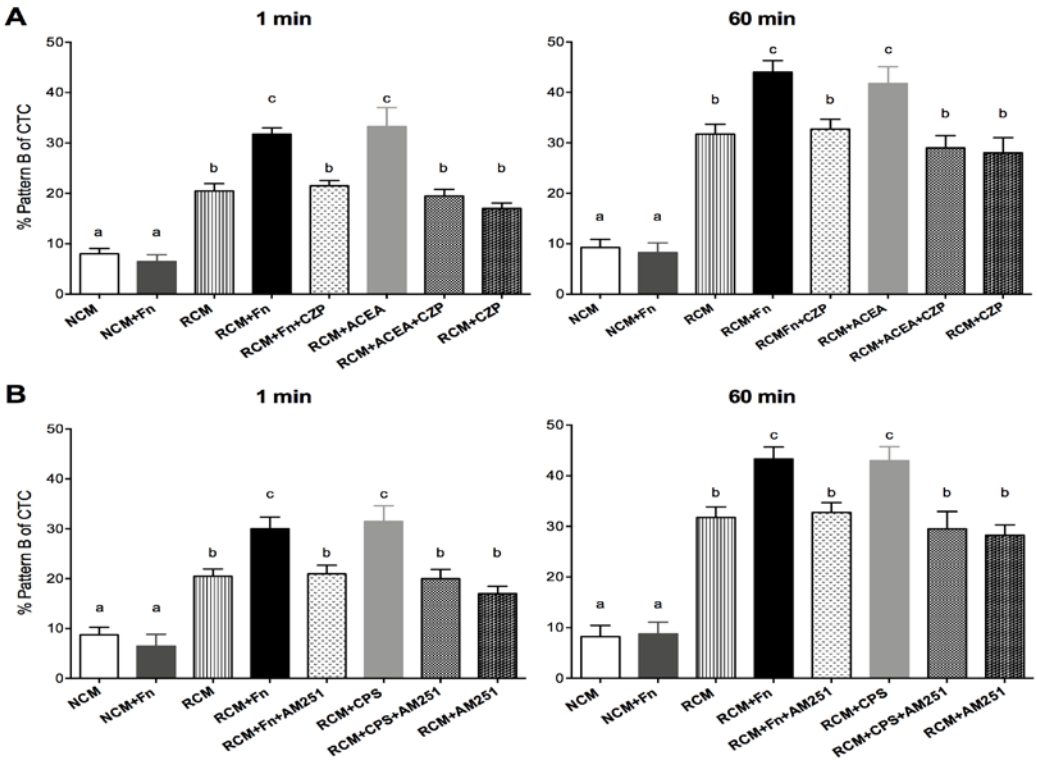


Figure 5. **FAAH activity is regulated by the cAMP/PKA pathway during human sperm capacitation induced by Fn**. (A) Human sperm were incubated in NCM, NCM+100 µg/ml Fn, RCM or RCM+100 µg/ml Fn. Simultaneously, sperm were pre-incubated in RCM with 10 µM H89 or 50 nM KT5720 in the presence of 100 µg/ml Fn. Thirty micrograms of protein per assay were used to determine FAAH enzymatic activity at 1 and 60 min. The bars represent enzymatic activity levels as determined by enzymatic ratio conversion. The results were obtained from nine independent experiments and are expressed as the mean±SEM. a≠b≠c $P<0.01$ (ANOVA, Tukey's multiple comparison test). (B) Human sperm were incubated in NCM, RCM or RCM+100 µg/ml Fn for 1 and 60 min. The bars represent the total cAMP concentrations measured using a commercial ELISA kit, as detailed in the Supplemental Materials and Methods section. The results were obtained from eight different donors and are expressed as the mean±SEM. *$P<0.05$; ***$P<0.001$ (ANOVA, Tukey's multiple comparison test). (C) Sperm were incubated in NCM (black grey circles) or RCM (black triangles) in the presence (dotted line) or absence of 100 µg/ml Fn (solid line) for 1 and 60 min. PKA activity was measured in sperm extracts, as indicated in the Supplemental Materials and Methods section. The results were obtained from five different donors and are expressed as the mean±SEM. *$P<0.05$ RCM+Fn vs RCM; ***$P<0.001$ RCM+Fn vs RCM (ANOVA, Tukey's multiple comparison test). (D) Aliquots of 200 µg of protein obtained from spermatozoa incubated in NCM or RCM with or without 100 µg/ml Fn were used for immunoprecipitation (IPP) for FAAH using magnetic beads (Dynabeads®) bound to recombinant protein G, after which SDS-PAGE was performed. The proteins were subsequently transferred to a PVDF membrane, which was probed with an anti-pPKAs antibody and then incubated with a biotinylated secondary antibody (anti-IgG). Finally, a streptavidin-peroxidase conjugate was added. The membrane was developed using an ECL reagent. As a loading control for IPP, immunoblotting using an anti-FAAH antibody under the same conditions as those described above was performed. Representative images of four independent experiments are shown.

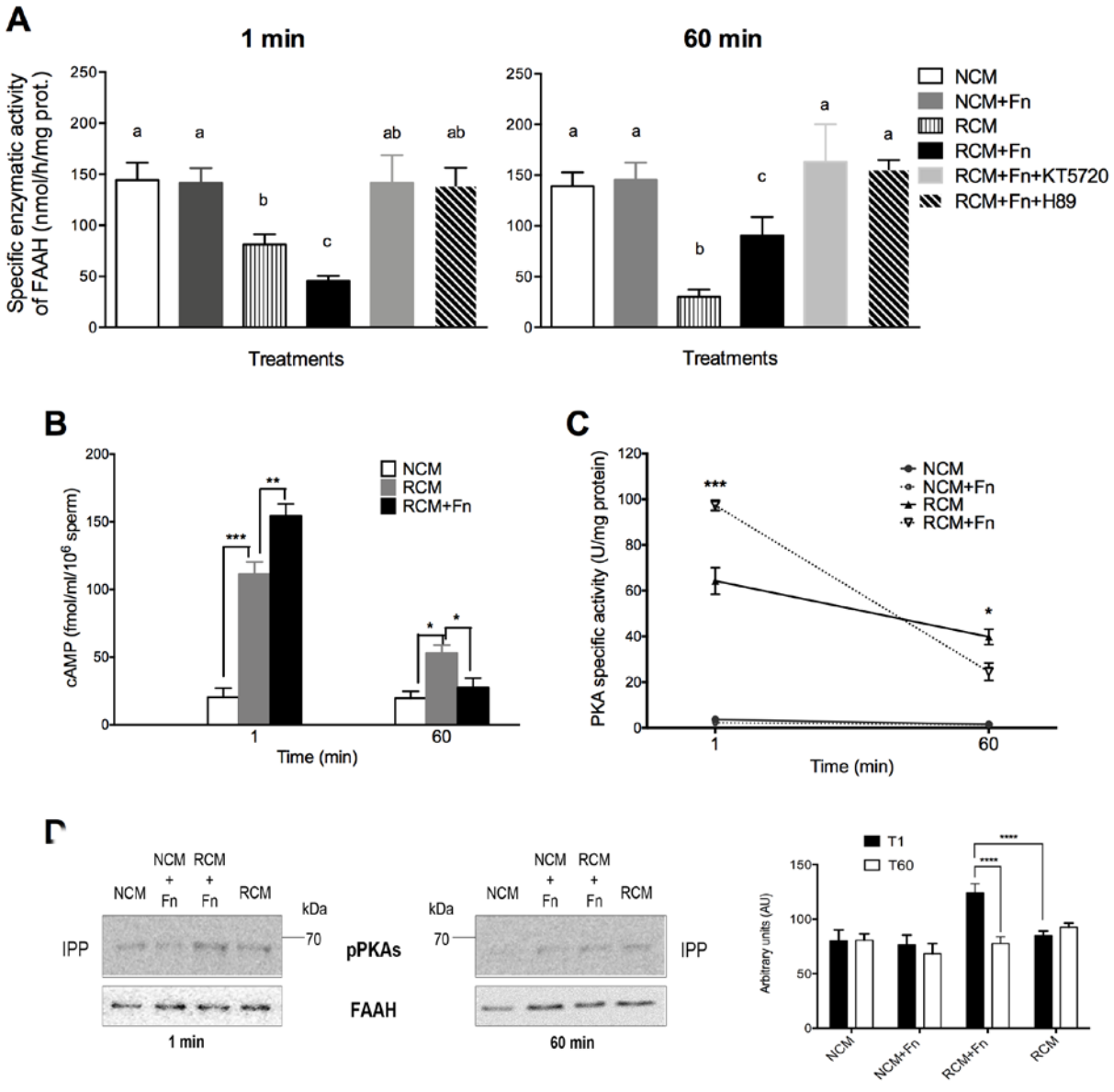
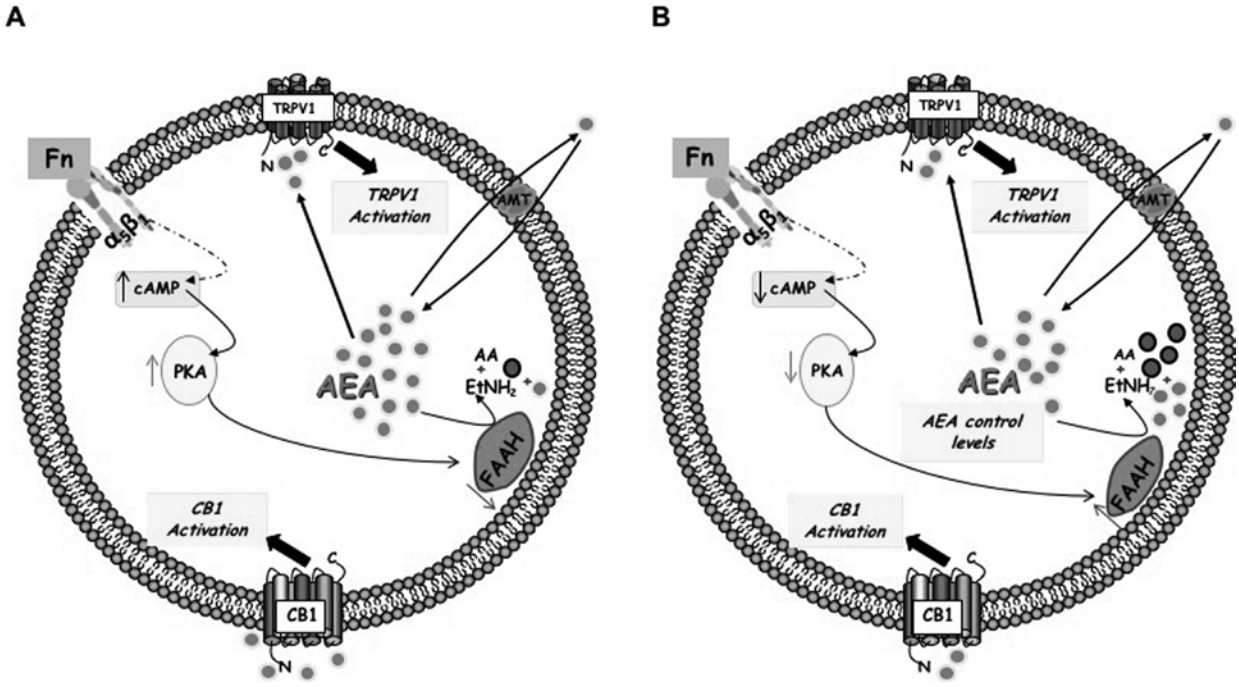


Figure 6. **Model of the participation of Fn in endocannabinoid system modulation during human sperm capacitation.** A) At 1 min, Fn-$\alpha_5\beta_1$ binding occurs and cAMP levels are increased, leading to PKA activation. PKA subsequently phosphorylates FAAH, thereby decreasing its activity and inducing a corresponding increase in AEA concentrations. AEA then activates the TRPV1 and CB1 receptors, promoting capacitation. B) At 60 min, Fn-$\alpha_5\beta_1$ binding reduces cAMP levels, which leads to decreased PKA activity. Subsequently, enzymatic activity of FAAH is increased, and AEA concentrations are decreased, preserving the AEA levels necessary for maintenance of capacitation.



Competing interest

The authors declare that they have NO affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest in the subject matter or materials discussed in this manuscript.